

**The effects of angiotensin-converting enzyme inhibitors as a pharmacological treatment for Dupuytren's disease**

By

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## Abstract

Dupuytren's disease is an acquired condition of the hand associated with fibrosis and contraction of the fascia of the palm, leading to stiffness and inability to properly flex and extend the digits. The disease is caused by both genetic and environmental factors. There is some evidence that Angiotensin II (ANGT II) may be associated with this disorder and that blockers of ANGT II may help in treating this disease. This study was designed to investigate the presence of ANGT II receptors 1 (AT1) and 2 (AT2) in myofibroblasts derived from primary tissue of 11 patients who had the disease. My hypothesis is that myofibroblasts will express angiotensin receptors and that these will primarily be of the AT1 receptor subtype.

Initially, the tissues were excised from patients who were undergoing surgery for Dupuytren's disease in the Department of Plastic Surgery at Royal Preston Hospital. The tissue from each patient was dissociated into myofibroblasts using collagenase. The myofibroblasts from each patient were cultured in a growth medium over a period of 8-10 days. The results show that it was possible to obtain successful growth curves for the myofibroblast following 8-10 days of cell culture. Some cultures produced better yields than others. The myofibroblasts were then stained and treated with specific polyclonal antibodies for the identification of AT1 and AT2 receptors. The results show that both AT1 and AT2 receptors were presented in myofibroblasts taken from all 11 patients. The results show a wider distribution of AT2 receptors compared with AT1 receptors. Samples of the myofibroblasts were employed to measure the contractile response and intracellular free calcium ( $\text{Ca}^{2+}$ ) concentrations. After numerous attempts using a collagen gel model to measure contraction, it was not possible to obtain any successful results. Similarly, fura loaded myofibroblasts show no signs of  $\text{Ca}^{2+}$  using a microspectrofluorometer to measure intracellular calcium. In conclusion, the results presented in this thesis have shown that it is possible to isolate tissues from patients with Dupuytren's disease and culture myofibroblasts successfully. Moreover, these myofibroblasts contain both AT1 and AT2 receptors. Further experiments are required to study the role of blocking AT1 and AT2 receptors excitation-contraction coupling process of the myofibroblasts.

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## **Declaration**

I declare that this thesis has been composed by myself and that, whilst registered as a candidate for the degree of Master of Science by Research, I have not been registered as a candidate for any other award or by any other awarding body.

A handwritten signature in black ink, appearing to read 'C Stephen', with a stylized flourish at the end.

Christopher Stephen

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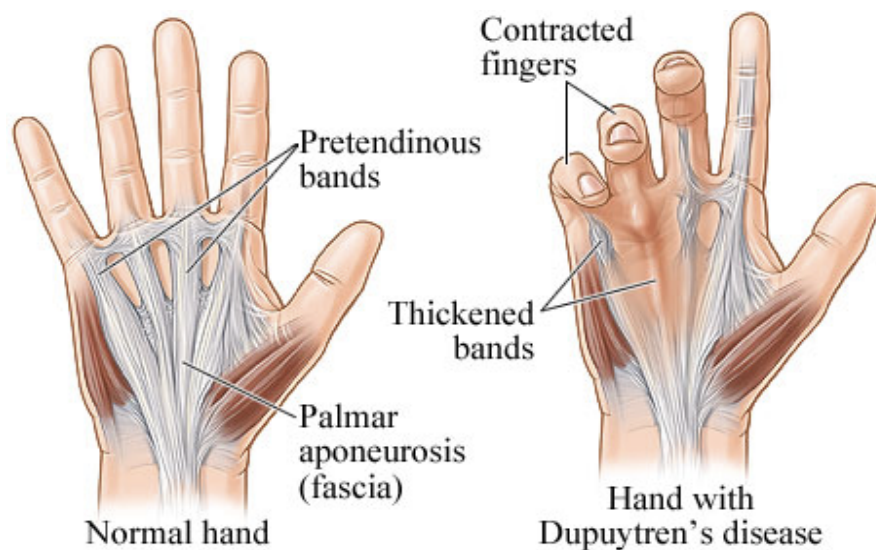


## **Chapter 1**

### **Introduction**

## Dupuytren's Disease

Dupuytren's disease (DD) is a multifactorial disorder involving genetic and environmental factors. It is a relatively common condition, which causes considerable morbidity primarily affecting the hands. DD is a fibrotic disease characterized by nodule formation and progressive scarring of the palmar fascia (Gudmundsson et al., 2003). Joint stiffness and a loss of full extension develop insidiously over decades (see Figure 1.1). As the scarring process progresses, nodules form on the palmar fascia and the finger gradually loses its flexibility, with contractures that pull the fingers into flexion at the metacarpophalangeal (MP) joint, resulting in significant loss of function (Shaw Jr, et al., 2007).



**Figure 1.1:** The palmar fascia in normal and Dupuytren's hands (Taken from Shaw Jr et al, 2007).

### History

The earliest reference to DD in medical literature was in 1614, when Plater described contractures of the ring and little fingers in a master mason in Basel. Cline described the disease in England in 1777 and proposed treatment by fasciotomy. Sir Astley Cooper, one of Cline's students, subsequently delivered lectures regarding the disease in London. Alexis Boyer wrote about finger contractures in the early nineteenth century in France. One of Boyer's students referred a patient with finger contracture to the surgeon, Guillaume Dupuytren, who operated on the patient and reported the case in a lecture in

1831. The lecture was subsequently published in *The Lancet* in 1834. Dupuytren described an association between the disease and manual labour, but more importantly, explained the exact anatomical features of the disease and suggested treatment with fasciotomy (Gudmundsson et al., 2003).

### **Aetiology**

DD is associated with diabetes mellitus, seizure disorders and chronic alcoholism. Despite many attempts to link DD with work or injury, no study has been conclusive (Liss and Stock, 1996). Recent reports have also associated DD with vibration injury (Liss and Stock, 1996). Although surgical treatment is routinely carried out with good effect, there is a significant rate recurrence (Shaw Jr et al., 2007). The precise mechanisms behind the disease are not fully understood. This has been a barrier to discovering an effective pharmacological therapy for the condition.

Although not fully understood, the aetiology is probably multi-factorial and has been shown to include familial and environmental factors (Burge, 1999). Recent research has implicated the *myofibroblast* cell in the pathogenesis of DD (Rudolph and Vande-Berg, 1991). This cell has also been shown to be involved in the pathogenesis of other fibro-proliferative disorders including keloid scarring, breast capsular contractures, myocardial infarct scarring, pulmonary fibrosis and renal fibrosis.

The epidemiology of DD is striking. The reported prevalence of DD ranges from 2% to 42%. This great variability is predominantly affected by ethnicity, age and gender, with the highest prevalence being found in men of northern European descent and rising steeply after the fourth and fifth decades (Ross, 1999). Men are, on average, six times more likely than women to present with the condition (Shaw Jr et al., 2007), with a reported male : female ratio ranging between 3.5:1 and 9:1 (Ross, 1999). Dupuytren's *diatheses* is a term used to describe patients with a strong predilection towards the disease (Shaw Jr et al., 2007), who often have bilateral as well as ectopic disease such as penile (Peyronie disease) and plantar (Lederhose disease).

## **Genetics**

The genetics of DD are not fully understood. Whilst a genetic influence has been mentioned in the literature since 1833 (Burge, 1999), the mode of inheritance has not yet been identified. Population studies in England and Norway have confirmed the suspicion that DD is more common in these areas (Ross, 1999), but similar studies have not been conducted in Asia or Africa, although anecdotal incidence suggests the disease to be very uncommon in these areas. Family studies have shown evidence suggestive of an autosomal dominant mode of inheritance. This was concluded by Skoog, who analyzed a number of studies, including his own (Skoog and Odelberg, 1948), in which they found a family history of DD to be present in 22 out of 50 cases. Subsequently, Ling (LING, 1963) obtained a positive family history in 8 out of 50 patients in Scotland. However, a further study has shown 34 of these patients have at least one affected family member. Although only 101 of the 832 relatives were affected, of the relatives aged 60 years or older, 53% of the men and 33% of the women were affected (Burge, 1999). The variation in prevalence, approaching 30% in individuals over the age of 60 in Norway (Burge 1999) as well as family clustering is evidence for an autosomal dominant mode of inheritance. However, many cases appear sporadically and with no family history. An autosomal recessive inheritance or a more complex mode of inheritance has been used to explain such cases, although there is no clear evidence for this. At present, although it is evident that an inherited susceptibility to DD exists, the condition is considered to be multifactorial. It has been suggested that the identification of susceptibility loci would be a worthwhile goal in understanding the disease as well as developing new and/or preventative treatments (Burge, 1999).

## **Cellular Level**

The clinically apparent DD nodule is composed of a number of smaller nodules that are non-encapsulated but surrounded by connective tissue and collagen bundles, the predominant cell being the myofibroblast (Shaw Jr et al., 2007). Early in the disease, the cells are spindle shaped. Later, when contracture is apparent, the cells are packed closely together and collagen production is evident.

Myofibroblasts exhibit the same morphological characteristics as normal fibroblasts but also express alpha smooth muscle actin ( $\alpha$ SMA) microfilaments, which allow them to display contractile behaviour (Gabbiani, 1994). Cell-to-cell attachments are also

apparent. They are involved in the normal wound healing process, where they produce collagen and extracellular matrix components shortly after the inflammatory phase. In subsequent remodeling and maturation phases, activated myofibroblasts stimulate wound contraction. However, when there is chronic myofibroblast activation, the synthesis of new collagen exceeds the rate of collagen degradation, leading to a net increase in total collagen as well as an increase in the force and rate of contraction. In Dupuytren's disease this leads to progressive thickening and contracture of the palmar fascia, resulting in fixed flexion and loss of function of the hand.

### **Angiotensin II**

In recent years it has become clear that Angiotensin II (ANGT II), plays not only a role in the regulation of body fluids, electrolytes and blood pressure, but also in cell proliferation and differentiation as well as local tissue repair and remodeling (Sun and Weber, 1996). ANGT II, the biologically active metabolite in the renin-angiotensin system, has been identified in many tissues including skin and subcutaneous tissue (Steckelings et al., 2004). There is now evidence that locally-manufactured ANGT II plays an important role in wound healing and fibrosis, with a stimulatory effect on fibroblasts and myofibroblasts (Takeda et al., 2004). Importantly, the presence of myofibroblasts containing ANGT II receptors has been demonstrated in Dupuytren's tissue (McKirdy et al., 2001). Several studies have demonstrated that angiotensin converting enzyme (ACE) inhibitors can reduce the amount of scarring and fibrosis in cardiac and renal animal models. Furthermore, in-vitro work has shown an increase in collagen production of cultured myofibroblasts with ANGT II, with a subsequent direct inhibitory effect of ACE inhibitors on these cells (McKirdy et al., 2001). These observations will form the basis of this study exploring the use of ACE inhibitors in Dupuytren's tissue.

### **The Renin-Angiotensin System (RAS)**

The renin-angiotensin system (RAS) has been extensively studied since being identified by Tiegerstadt and Bergman in 1898. It has historically been described as a hormonal axis, participating significantly in the regulation of fluid and electrolyte balance to maintain cardiovascular homeostasis (Campbell, 1987).

The pathophysiological implications of the system have been the focus of much

attention, and inhibitors of the RAS such as angiotensin-converting enzyme (ACE) inhibitors and angiotensin (ANGT) II receptor blockers have become important clinical tools in the treatment of cardiovascular and renal diseases such as hypertension, heart failure, and diabetic nephropathy (Paul et al., 2006).

It functions as an unusual endocrine axis in which the active hormone, ANGT II, is formed in the extracellular space by sequential proteolytic cleavage of its precursors. The secretion of the enzyme renin initiates the pathway, which is activated by several stimuli including volume depletion and sodium concentration (Atlas, 2007).

The RAS cascade begins with the synthesis of renin by the juxtaglomerular cells that line the afferent arteriole of the renal glomerulus (Barrett 2010). The regulation of renin secretion takes place via renal baroreceptor mechanisms, renal NaCl concentration, sympathetic nerve stimulation and negative feedback from ANGT II on the juxtaglomerular cells. Renin acts by cleaving the precursor angiotensinogen to form the biologically inert ANGT I (Paul, 2006)). The primary source of circulation angiotensinogen is the liver. ANGT I is rapidly hydrolyzed by the membrane-bound angiotensin-converting enzyme to form the biologically active ANGT II (Barrett, 2010).

The multiple actions of the octapeptide ANGT II are mediated by specific receptors on the membranes of target cells (Johnston 1990). It is a potent vasoconstrictor, with action directly on vascular smooth muscle as well as stimulating the sympathetic nervous system, which can also increase heart rate. It acts on cardiac myocytes to increase contractility by prolonging the cardiac action potential. It also stimulates the release of catecholamines from the adrenal medulla, which can also increase total peripheral resistance (Brunton et al., 2005). Other actions on the cardiovascular system include hypertrophy of cardiac cells and increased synthesis and degradation of collagen by cardiac fibroblasts (Brunton et al., 2005).

As with the cardiovascular system, the effects of ANGT II on the kidney are multifaceted. It increases sodium reabsorption via the stimulation of aldosterone release from the adrenal cortex as well as via a direct effect on renal tubules. It can also reduce renal blood flow by directly constricting the renal vascular smooth muscle and by stimulating the central nervous system. This results in markedly decreased renal excretory function

(Brunton, 2005). ANG II also reduces renin secretion through negative feedback.

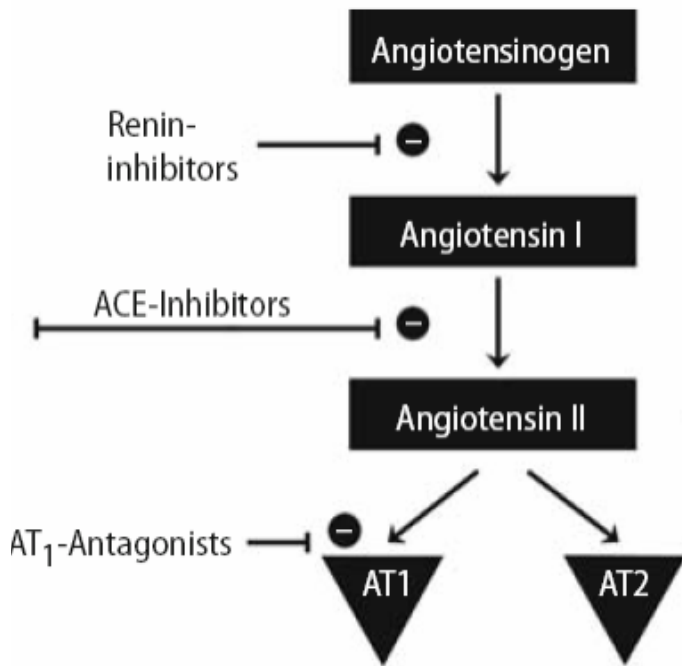
ANG II also reduces the sensitivity of the baroreflex in the brain, which potentiates the vasopressor effect (Barrett, 2010).

## **Renin**

Renin, a proteolytic enzyme, is synthesised, stored and secreted into the renal arterial circulation by the granular juxtaglomerular cells lying in the walls of afferent arterioles (Johnston, 1990). Renin is stored in granules within these cells and secreted by exocytosis (Friis, 1999). As with many proteins, renin is initially synthesised as an inactive precursor molecule that is subsequently converted into the active form. The concentration of inactive prorenin in the circulation is approximately tenfold greater than the active enzyme (Brunton, 2005). Active renin consists of 2 polypeptide chains linked by a disulphide bond. Renin acts only on its specific substrate angiotensinogen to form ANG I, a decapeptide (Johnston, 1990).

## **Angiotensin Converting Enzyme (ACE)**

ACE is a membrane-bound glycoprotein with an apparent molecular weight of 170 kDa. It is localized on the plasma membranes of various cell types, including vascular endothelial cells, microvillar brush border epithelial cells (e.g., renal proximal tubule cells), and neuroepithelial cells (Atlas, 2007). The highest concentration of ACE is on the surface of pulmonary endothelial cells (Paul, 2006). ACE is non-specific and cleaves dipeptides, including the vasodilator peptides bradykinin and kallidin, to inactive metabolites. Thus, functionally, the enzymatic actions of ACE potentially result in increased vasoconstriction and decreased vasodilation. Although it is unspecific, ACE is the only known enzyme to convert ANG I into active ANG II (Dale, 2012).



**Figure 1.2:** Flow diagram showing the Renin-Angiotensin System

(Taken from the Clinical Research Competence Centre, University Hospital Erlangen, Germany)

### Angiotensin Receptors

The effects of ANG II are exerted through specific angiotensin receptors. The two major receptor subtypes are the AT<sub>1</sub> and AT<sub>2</sub> receptors (Figure 1.2). These have been identified using specific angiotensin receptor ligands and cloning these receptors. Most of the known biological effects of ANG II are mediated by the AT<sub>1</sub> receptor (Brunton, 2005). With advent of more sophisticated methods, further analysis of the AT<sub>1</sub> receptor has identified two specific subtypes - AT<sub>1A</sub> and AT<sub>1B</sub> (Sun, 1996a). The AT<sub>1A</sub> subtype is predominant in vascular smooth muscle, lung, heart and hypothalamus tissue whilst the AT<sub>1B</sub> subtype predominates in uterus, adrenal gland and pituitary tissue. Two further ANG II receptors have been proposed (AT<sub>3</sub> and AT<sub>4</sub>), but this is yet to be fully understood.

The AT<sub>1</sub> receptor is the predominant receptor found in the vascular smooth muscle, heart, adrenal cortex, liver, kidney and some regions of the brain and is responsible for most of the known functions of the renin-angiotensin system in both physiological and pathophysiological conditions (Guimarães, 2005). Importantly, stimulation of AT<sub>1</sub>



receptors by ANG II has been shown to influence growth and injury response in various organs (Weber, 1999). The AT1 receptor belongs to the G protein-coupled receptor family and is coupled to several effector systems involved in cellular growth, synthesis and contraction (Guimarães, 2005).

Less is understood regarding the AT2 receptor. This receptor is widely distributed in fetal tissues, whereas in adults this distribution is far more restricted (Brunto, 2005). AT2 receptor is predominant in uterine smooth muscle, ovary, adrenal medulla and some brain regions (Guimarães, 2005). Skin, connective tissue and gastrointestinal tissue have also been shown to express this receptor, and it appears to have a role in cell growth and response to injury.

Evidence is accumulating that AT2 receptors have a significant role in the regulation of cardiovascular function, including some studies that suggest AT2 receptors activate a vaso-dilatatory pathway, which counteracts the vasoconstrictor action of ANG II exerted through the AT1 receptor (Guimarães, 2005).

### **Tissue (Local) Renin-Angiotensin System (RAS)**

The traditional view of the RAS is that of a classical endocrine system whereby the various circulating components result in the delivery of ANG II to its target organs via the bloodstream, producing a physiological response (Brunton, 2005). In addition to the circulating RAS, there is much evidence for the ability of tissues such as the brain, pituitary, heart and kidney to produce ANG II (Johnston, 1990; Campbell 1987). Numerous studies have also shown that the requisite components of the RAS, such as renin, angiotensinogen and ACE are present in such tissues (Dinh, 2001). Furthermore, tissue repair has been studied in various organs of the rat including the heart, pericardium, skin and kidney. In these tissues ACE and ANG II binding density was measured. High-density ACE and ANG II binding was shown in areas of fibrosis in contrast to non-injured tissue, where binding density was low (Weber, 1997). Using a rat model of myocardial infarct, tissue concentration of ANG II was increased in areas of repair. It is now understood that ANG II plays a significant role in wound healing, fibrosis and extracellular matrix homeostasis (Allen 2000). The concept of extracellular matrix homeostasis is that fibroblast-derived ANG II regulates the elaboration of

transforming growth factor beta (TGF- $\beta$ ), a cytokine responsible for connective tissue formation at normal and pathological sites of collagen turnover (Weber, 1999). Several studies have demonstrated a role for ANG II in tissue fibrosis and wound healing, particularly in cardiac infarct tissue (Weber, 1997; Sun, 2000; Sun, 1996a; Sun, 1996b; Sun, 1997; Weber, 2000). Such studies identify the myofibroblast to be the key cell involved in this process.

### ***ACE Inhibitors***

In order to block the effect of ANG II, one can either prevent the conversion of ANG I to ANG II or block the AT receptors on which ANG II acts. ACE inhibitors are highly selective drugs suppress the synthesis of ANG II, although ACE has other substrates which may induce parallel effects unrelated to ANG II levels (ie bradykinin) (Brunton, 2005).

Many ACE inhibitors have been synthesised. These can be classified into three groups based on chemical structure:

- 1: sulphydryl-containing ACE inhibitors – structurally related to Captopril
- 2: dicarboxyl-containing ACE inhibitors – structurally related to Enalapril
- 3: phosphorus-containing ACE inhibitors – structurally related to Fosinopril

Several ACE inhibitors are currently available for clinical use, and these differ with regard to three properties (Brunton, 2005):

- 1: potency
- 2: whether AE inhibition is a direct effect of the drug itself or the effect of the active metabolite
- 3: pharmacokinetics (absorption, half-life, tissue distribution, elimination)

*Captopril* was the first ACE inhibitor to be marketed. It is a potent ACE inhibitor which, given orally, is rapidly absorbed and has a bioavailability of about 75%. Peak plasma concentrations occur within 1 hour and it is cleared rapidly with a half-life of 2 hours. As

with most ACE inhibitors, it is cleared by the kidneys and excreted mainly in the urine (Brunton, 2005).

*Enalapril* maleate is a prodrug that is hydrolysed by esterases in the liver to produce the active dicarboxylic acid, enalaprilat. This is a potent ACE inhibitor that is rapidly absorbed with an oral bioavailability of 60%. Peak concentrations occur within 1 hour, peaking at 3-4 hours. The plasma half-life is only 11 hours and excretion is primarily renal (Brunton, 2005).

*Lisinopril* a non-sulphur-containing pro-drug. It is the lysine analogue of enalaprilat. In vitro, it is a slightly more potent ACE inhibitor than the latter. When taken orally, it is absorbed slowly, variably and incompletely (30%). Its plasma half-life is 12 hours and it is excreted by the kidney. Lisinopril does not accumulate in tissues (Atlas, 2007).

### ***The Extracellular Matrix (ECM)***

The extracellular matrix (ECM) is the largest component of normal skin (Schultz, 2005). It is this component that gives skin properties such as elasticity, compressibility and tensile strength. In acute wounds the provisional wound matrix, containing fibrin and fibronectin, acts as a scaffolding to direct cells to the site of injury as well as to stimulate cell differentiation and the production of new ECM. The synthesis of ECM is a key feature of wound healing. Specifically, ECM is actively involved in both cellular and extracellular events that lead to fibrosis (Wight, 2011). The constituents of ECM include polysaccharides, water, collagen and proteins. The 2 main classes of ECM molecules are fibrous structural proteins and glycosaminoglycans.

Fibrous structural proteins give the ECM strength and they include collagens, elastin, laminin. In contrast, proteoglycans consist of multiple glycosaminoglycan chains that branch from a linear protein core. These are large, highly hydrated molecules that help cushion cells in the ECM (Schultz, 2005).

Collagen is the largest class of fibrous ECM molecule and is responsible for tensile strength. There are multiple types of collagen depending on the type and arrangement of protein chains; in the dermis collagen is mainly type I and type III. Molecules are rod

shaped in a staggered, cross linked side-to-side arrangement (Schultz, 2005). Type IV collagen associates with other ECM molecules to form the basement membrane (Brauer, 1989).

Laminin is a large, cross-shaped molecule which binds Type IV collagen and other proteins via integrin receptors. It is therefore a multi-adhesive matrix protein which forms bridges between cells and the basement membrane (Frantz, 2010). Elastin is the main component of elastic ECM fibres, which consist of adjacent cross-linked elastin molecules. This gives certain tissue the ability to recoil following physiological stretch (Frantz, 2010).

Fibronectin is a multifunctional glycoprotein with many different binding sites for collagen and fibrin molecules, allowing it to link together different components of ECM for cell adhesion (Wight, 2011). Also, the binding of fibronectin molecules to certain (integrin) cell receptors leads to stimulation of signalling pathways that promote cell attachment, migration and differentiation, thus allowing communication between cells and the ECM (Schultz, 2005).

Glycosaminoglycans (GAG) are composed of polysaccharide chains and are highly negatively charged. This means that they are strongly hydrophilic as they attract osmotically active  $\text{Na}^+$ , causing large amounts of water to be drawn into their structure (Kim, 2011). As a result, they occupy a huge volume relative to their mass and causes turgor, which allows the ECM to withstand compression forces. Hyaluronic acid is a GAG found in growing or damaged tissue, which stimulates angiogenesis (Frantz, 2010).

Proteoglycans retain water and form a gel-like substance through which ions, hormones and nutrients can move freely. They also appear to have a role in regulating cell signalling (Suki, 2008). For example, heparin sulphate chains of proteoglycans bind fibroblast growth factors and aid binding of such factors to their receptors. Proteoglycans can also bind chemokine molecules such as TGF- $\beta$  (Schultz, 2005).

TGF- $\beta$  directly increases fibrosis and scar formation by increasing the expression of collagen and fibronectin while reducing the expression of MMPs (matrix metalloproteinases) (Leask, 2004).

## **The Process of Wound Healing**

The phases of wound healing are overlapping but biologically distinct. These include an initial inflammatory phase, which includes haemostasis, removal of devitalised tissue and prevention of infection. This is followed by a proliferative phase, where the provisional wound matrix is remodelled and replaced by scar tissue. The final phase is remodelling, during which tissue maturation occurs and wound strength is maximised (Diegelmann, 2004).

### **Haemostasis and Inflammatory Phase: 24-48hrs**

Initial injury causes endothelial disruption and release of proteolytic enzymes. Haemorrhage ensues and a platelet plug adheres to exposed type II collagen following haemostatic cascades (Witte, 1997). Within the platelet plug are growth factors (PDGF, TGF- $\alpha, \beta$ ) and inflammatory cytokines (fibrinogen, fibronectin, vWF). Initial vasoconstriction is followed by vasodilation due to inflammatory mediators (histamine, kinins, complement). Vasodilation results in vessel engorgement and plasma exudation into the damaged area. PDGF secreted by platelets during thrombus formation acts as a chemo-attractant for macrophages, which remove bacteria and debris. Macrophages are also a source of growth factors including TGF- $\beta$ , which is a chemo-attractant for fibroblasts, which migrate into the wound by forming cell-matrix contacts with matrix proteins (fibronectin, vitronectin, fibrinogen). The migration of fibroblasts into the wound signals the start of the proliferative phase. Growth factors and proteins in the provisional wound matrix stimulate fibroblasts to synthesise collagen and extracellular matrix components (Li, 2007; Diegelmann, 2004; Witte, 1997a).

Within 12 hours, neutrophils and monocytes migrate to the wound (attracted by PDGF, TGF  $\beta$ , complement proteins, fibrin degradation products and leukotrienes). Initially neutrophils adhere to the vascular endothelial cell wall releasing elastase and collagenase, which allows migration through the basement membrane and into the wound matrix where they phagocytose and kill bacteria. The neutrophil response declines after a few days. At this stage monocytes are attracted via soluble mediators and bind the extracellular matrix via integrin receptors. Here they differentiate into macrophages and have 3 main functions (Diegelmann, 2004).

- Phagocytosis of bacteria, devitalised tissue and depleted neutrophils
- Production of collagenase and elastase, facilitating proteolytic destruction of devitalised tissue
- Release of soluble mediators (TNF- $\alpha$ , TGF- $\beta$ , PDGFs, IL-1, IL-6), which recruit and activate fibroblasts and promote angiogenesis (Li, 2007).

### **Proliferative Phase: Day 3 up to 4 weeks**

The provisional wound matrix is remodelled and replaced by scar tissue, with subsequent increase in the tensile strength of the wound. This consists of collagen, proteoglycans and elastin fibres. In order for this to take place, epithelial cells, fibroblasts and vascular endothelial cells migrate to and proliferate in the wound site. Fibroblasts actively produce collagen and lay down extracellular matrix to strengthen the wound in contrast to normal dermal fibroblasts, which are typically sparse and quiescent (Stone, 2006).

Collagen Production: Normal dermis contains mainly type I collagen. During early wound healing fibroblasts secrete type III collagen, which is replaced by type I collagen from week 2 onwards. Collagen synthesis peaks at 5-7 weeks with maximal levels present within 6 weeks, although increased synthesis and degradation continue for up to 1 yr. The early collagen is disorganised and wound tensile strength is poor. Granulation tissue consisting of capillary loops and surrounding collagen fibres is formed (Irwin 1981).

### **Remodelling Phase**

This is the longest phase of wound healing and is believed to last from 21 days up to 1 year. It commences once the wound defect has been filled with granulation tissue and it has been re-epithelialized by migrating keratinocytes. The precise mechanisms by which this takes place are not fully understood but during this period wound contraction, collagen remodelling and a progressive decrease in scar vascularity occur (Witte 1997a).

During collagen remodelling type III collagen is slowly degraded and replaced by type I collagen. There is also increase collagen cross-linking and the tensile strength of the wound subsequently increases. Wound contraction is produced by myofibroblasts (fibroblasts with actin filaments capable of matrix contraction).

### **The Myofibroblast**

Myofibroblasts are specialised cells that share the morphological characteristics of fibroblasts and smooth muscle cells (Gabbiani, 1992). *Fibroblasts* are elongate cells with a slender, smooth nucleus, scattered mitochondria, a large Golgi apparatus and numerous endoplasmic reticulum. They contain actin microfilaments, but these are concentrated beneath the plasma membrane as a meshwork rather than in bundles. In contrast, in *smooth muscle cells*, the actin molecule is organised into bundles that fill most of the cell. These bundles are oriented along the long axis of the cell, and the cells possess numerous specialised attachment sites. Smooth muscle cells also have a poorly developed Golgi apparatus and endoplasmic reticulum (Tomasek, 1999).

Myofibroblasts are large, spindle-shaped cells with nuclear indentations. The cells are interconnected and linked to the extracellular matrix through a complex system of microfilaments bridging the plasma membrane and extracellular fibronectin. The predominant differentiating feature between myofibroblasts and fibroblasts is the presence bundles of actin microfilaments, although there are less bundles in myofibroblasts than in smooth muscle cells (Tomasek, 1999). The bundles are composed of actin, myosin and associated proteins. There are six different recognised isoforms of actin, classified according to their pattern of expression as follows: striated muscle - two muscle actins ( $\alpha$ -skeletal and  $\alpha$ -cardiac), smooth muscle – two muscle actins ( $\alpha$ -smooth muscle actin and  $\gamma$  smooth muscle actin) and every other cell type – two non-muscle actins ( $\beta$ -actin and  $\gamma$ -actin). Whilst fibroblasts contain only  $\beta$ -actin and  $\gamma$ -actin, myofibroblasts also express  $\alpha$ -smooth muscle actin, although this expression is transient (Tomasek, 1999).

The myofibroblast is a key cell for the connective tissue remodeling that takes place during wound healing and fibrosis development. In 1971, Gabbiani first described the

myofibroblast in the granulation tissue of an open wound using electron microscopy as an intermediate cell between the fibroblast and the smooth muscle cell (Gabbiani, 1998). The myofibroblast has been identified both in normal tissues, particularly in locations where mechanical force is necessary, and in pathological tissues such as hypertrophic scarring, fibromatoses and fibrocontractive diseases (Desmoulière, 2005). Initially, it was postulated myofibroblasts play a contractile role due to the coincidence of the presence of myofibroblasts in such tissues. However, direct proof of the presence and activity of contractile elements in these cells was possible through advances in techniques to localize and quantify cytoskeletal and contractile proteins (Gabbiani, 1992). It is now accepted that fibroblast/myofibroblast transition begins with the appearance of the protomyofibroblast, which subsequently differentiates into a myofibroblast with stress fibers containing alpha-smooth muscle actin (Desmoulière, 2005). In theory, this allows the cell to generate a contractile force. Myofibroblast differentiation is a complex process, regulated by TGF  $\beta$ 1, an extracellular matrix component (a variant of cellular fibronectin), as well as the presence of mechanical tension (Desmoulière, 2005).

### **Myofibroblast Differentiation**

Myofibroblasts have been observed in several types of tissue in addition to granulation tissue as originally identified. These include skin, breast, kidney, heart, eye, liver, lung, GI tract, bone marrow, joints, uterus and testicle (Klingberg, 2012). The presence of myofibroblasts in Dupuytren's disease tissue has been observed for a considerable amount of time (Chiu, 1978), the regulation of which is not yet fully understood. This will be addressed in more detail later in this thesis. It is important, however, to understand the basic mechanisms behind myofibroblast differentiation in general.

The process of wound healing was described earlier. The initial stage of this process involves platelet degranulation, which releases cytokines including platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and transforming growth factor  $\beta$  (TGF  $\beta$ ). PDGF is a chemoattractant for macrophages, which produce fibroblast growth factor (FGF) and tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ). Collectively these cytokines are all chemoattractant for fibroblasts, which subsequently migrate to the injured site (Klingberg, 2012). It is believed that more specific migration factors are present but this is not yet fully understood.



TGF- $\beta$ 1 is thought to be an important factor in promoting myofibroblast differentiation. One study, where TGF  $\beta$ 1 was administered subcutaneously to rats, resulted in myofibroblast-containing granulation tissue formation (Desmoulière, 2005). The cells expressed  $\alpha$ -smooth muscle actin granules, the formation of which is deemed to be synchronous with myofibroblast activation. TGF- $\beta$ 1 was also demonstrated to induce  $\alpha$ -smooth muscle actin expression in quiescent and growing fibroblast populations (Desmoulière, 1993).

### **Myofibroblasts in Wound Healing and Tissue Repair**

Myofibroblasts feature in injured or healing tissue such as the kidney, heart and skin. These cells play a role in synthesis and contraction of the extracellular matrix. Several growth factors are believed to be involved in their differentiation, as well as matrix components and mechanical tension (Desmoulière, 2005). One such growth factor, transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1), has been shown to influence their conversion. TGF- $\beta$ 1 is a ubiquitous polypeptide growth factor thought to have a central role in fibrotic conditions (Bisson, 2009). It has been shown to increase collagen deposition in granulation tissue myofibroblasts (Gabbiani, 1998) as well as fibroblasts in Dupuytren's disease (Alioto 1994). TGF- $\beta$ 1 expression has also been linked to the rennin-angiotensin system (Campbell, 1997).

Both fibroblasts and myofibroblasts have been shown to exhibit AT1 and AT2 receptors for ANGT II. As discussed earlier, an increase in ANGT II levels is clearly observed in the progression of fibrosis (Sun 1997a). Furthermore, the administration of ANGT II has been shown to increase the progression of fibrosis in the rat heart and kidney (Sun 1993).

## **Tissue culture**

Tissue culture is the growth of tissue or a cell separate from the organism or in vitro growth in Nutrient medium. This typically facilitated via use of liquid (Growth media), semisolid growth media such as broth and agar. Tissue culture commonly refers to the culture of animal cells and tissues.

In General, there are two types of cell culture

- a. Primary –derived from a living organism.
- b. Cell lines –Stable established cells which proliferate in culture.

Both types of culture employ similar practical sterile techniques.

Biological research method in which tissue fragments (a cell, a population of cells, or all or part of an organ) are sustained in an artificial environment for examination and manipulation of cell behaviour.

It has been used to study normal and abnormal cell structure biochemical, genetic, and reproductive activity; metabolism, functions, and reactions to physical, chemical, and biological agents (e.g., drugs, viruses).

A sample of the tissue is spread on or in a culture medium of biological (e.g., blood serum or tissue extract), synthetic, or mixed origin having the appropriate nutrients, temperature, and pH for the cells being incubated. The results are observed with a microscope, sometimes after treatment (e.g., staining) to highlight particular features.

Work with tissue cultures has helped identify infections, enzyme deficiencies, and chromosomal abnormalities, classify brain tumours and formulate and test drugs and vaccines.

Cultures have been used to investigate fundamental processes of growth and development in both normal and abnormal tissues. One finding has been that normal cells undergo an aging process, retaining their ability to multiply readily for only 50 to 100 generations, after which the rate decreases markedly. This study employed the tissue culture technique to grow myofibroblasts.

## **Immunohistochemistry**

Immunohistochemistry is the localization of antigens or proteins in tissue sections by the use of labelled antibodies as specific reagents through antigen-antibody interactions that are visualized by a marker such as fluorescent dye, enzyme, or colloidal gold.

### **Antibodies**

Antibodies belong to a group of proteins called immunoglobulins (Ig). The immunoglobulins comprise five major classes (in order of decreasing quantity found in plasma): IgG, IgA, IgM, IgD and IgE. Each is composed of two identical heavy chains and two identical light chains. IgG and IgM are the most utilised immunoglobulins for immunohistochemistry (Atassi et al., 1984).

Polyclonal antibodies are produced by different cells, and in consequence, are immunohistochemically dissimilar; they react with various epitopes of the antigen against which they are raised. The most frequently used animal for the production of polyclonal antibodies is the rabbit. The popularity of rabbits for this purpose is attributed to their ease of maintenance (Boenisch, 2001).

Monoclonal antibodies are the product of an individual clone of plasma cells. Antibodies from a given clone are immunochemically identical and react with a specific epitope on the antigen against which they are raised. Mice are the most commonly used animals for the production of monoclonal antibodies (Harboe, 1983).

### **Direct Method:**

The direct staining method is a one- step staining method, and involves a labeled antibody (i.e. FITC conjugated antiserum) reacting directly with the antigen in tissue sections. This technique utilizes only one antibody and the procedure is short and quick. However, it is insensitive due to little signal amplification and is rarely used since the introduction of the indirect method. This is the oldest immunostaining technique.

### Indirect Method:

Indirect method involves an unlabeled primary antibody (first layer), which reacts with tissue antigen, and a labeled secondary antibody (second layer), which reacts with primary antibody. (Note: The secondary antibody must be against the IgG of the animal species in which the primary antibody has been raised). This method is more sensitive due to signal amplification through several secondary antibody reactions with different antigenic sites on the primary antibody. In addition, since one labeled second layer antibody can be used with many first layer antibodies (raised from the same animal species) to different antigens.

The second layer antibody can be labeled with a fluorescent dye such as FITC, rhodamine or Texas red, and this is called indirect immunofluorescence method. The second layer antibody may be labeled with an enzyme such as peroxidase, alkaline phosphatase or glucose oxidase, and this is called the indirect immunoenzyme method.

Furthermore, a three-step indirect method utilizes a third antibody layer, which further amplifies the signal (Boenisch, 2001).

### **Calcium ( $\text{Ca}^{2+}$ )**

Calcium is the most important physiological cation in cellular regulation. It is the trigger, the promoter and the regulator and moreover, a ubiquitous intracellular signalling molecule which controls a wide range of cellular processes including secretion, membrane transport, contraction, cell proliferation, gene transcription and even cell death. In un-stimulated cells, the free intracellular calcium concentration  $[\text{Ca}^{2+}]_i$  is between 50–100 nM. In order to maintain this low resting level, a variety of pumps and uptake systems are present in the plasma membrane and in intracellular organelles to buffer ( $\text{Ca}^{2+}$ ). Calcium mobilisation is dependent upon intracellular calcium stores as well as extracellular calcium medium  $[\text{Ca}^{2+}]_o$ . An increase in cellular calcium originates from two major sources:

- a. The release of  $\text{Ca}^{2+}$  from intracellular stores (ER), which is rapid. Calcium release from the ER after  $\text{IP}_3$  generation is crucial for exocytosis. Similarly the release of  $\text{Ca}^{2+}$  from SR is crucial in muscle contraction.
- b. The influx of  $\text{Ca}^{2+}$  from extracellular source with the aid of  $\text{IP}_3$  by the depletion of an intracellular pool.

Both of these mechanisms give rise to high-localised ( $\text{Ca}^{2+}$ ) signals. Many agonists can promote an influx of  $\text{Ca}^{2+}$  from the extracellular medium by the following processes:

- a. Opening of voltage-operated  $\text{Ca}^{2+}$  channels
- b. Phosphorylation of  $\text{Ca}^{2+}$  channel proteins by cAMP and cGMP.
- c. Receptor-operated  $\text{Ca}^{2+}$  channels in which modulation of channel activity does not involve cytosolic signal molecules but a direct control by receptor channel coupling G- proteins.

**Working hypothesis:** Angiotensin receptors may be involved in the aetiology and development of Dupuytren's disease and ACE inhibitors may play a role in the pharmacological treatment of the disease.

**Main Aim:** The main aim of this study was to isolate and culture myofibroblasts from primary tissues from patients who have Dupuytren's disease and to investigate the role of angiotensin receptors in the development and treatment of the disease.

**Specific aims:**

1. To undertake a thorough literature review in the area.
2. To isolate the tissues from patients and to culture them into myofibroblasts.
3. To study the morphology of the myofibroblasts and to undertake immunohistochemical staining for the presence and distribution of angiotensin receptors.
4. To study the contraction of the myofibroblasts using a collagen gel system
5. To measure intracellular free calcium concentration  $[\text{Ca}^{2+}]_i$  in the myofibroblasts using fura-2 loaded cells.
6. To analyse the data and write up the MSc by research

## **Chapter 2**

### **Materials and Methods**

## Materials

**Patients:** Eleven patients from Royal Preston Hospital  
**Tissues:** Isolated skin tissues from all 11 patients  
**Reagents:** Trypsin, collagenase  
**Antibodies:** Anti-AT1 receptor antibody produced in rabbit (Anti-AGTR1, Sigma Aldrich)  
Anti-AT2 receptor antibody produced in rabbit (Anti-AGTR2, Sigma Aldrich) and  
Anti-Actin,  $\alpha$ -Smooth Muscle antibody produced in mouse (Sigma Aldrich A5228).

**Culture media:** DMEM, foetal calf serum, Ham's F10

**Chemicals:** Analar grade chemicals, DMSO

**Immunohistochemistry:** Vectastain® Universal Elite ABC Kit

**Drugs:** Angiotensin II  
Lisinopril  
Penicillin  
Streptomycin  
Amphotericin  
Glutamine  
[Val<sup>5</sup>]-Angiotensin II acetate salt hydrate  
Irbesartan  
S-(+)-PD 123177 trifluoroacetate salt hydrate  
1-Methylhydantoin

## Methods

### Tissue Harvesting

Patients who were scheduled for surgery to remove Dupuytren's tissue were identified on the waiting list at Royal Preston Hospital. Verbal and written information was provided to patients at least one day prior to surgery (see Appendix). Consent to use tissue for research was obtained from LREC (see Appendix) and tissue specimens were transferred from operating room to the hospital cell culture laboratory in the collection medium. Each patient gave written consent to use their tissue for research (see Appendix).

## **Cell Culture**

Dupuytren's tissues were isolated and then stored in collection medium at 4°C for up to 48 hours after harvest. The biopsy was transferred to a Petri dish along with fresh collection medium (Dulbecco's Modified Eagle Medium, Penicillin, Streptomycin, Amphotericin). Using a sterile forceps and scalpel, any obvious non-Dupuytren's tissue was removed. The tissue was then transferred to a fresh Petri dish and macerated into approximately 1mm x 1mm fragments using a scalpel.

The fragments were then transferred into a universal container with 10-15 ml of collection medium, allowing the larger pieces to settle and discarding the supernatant. The fragments were then re-suspended in a centrifuge tube with 10 ml complete growth medium (Dulbecco's Modified Eagle Medium, L-Glutamine solution, Ham's F10, foetal calf serum). 1 ml of collagenase solution (Collagenase type 1a, Hank's Balanced Salt Solution) was added and the tubes were left to incubate at 37°C for up to 12 hours, or when disaggregation was near completion. The solution was pipetted several times to aid further disaggregation and then centrifuged for 5 min at 1500 RPM). The supernatant was removed and the cells were re-suspended in 10 ml complete growth medium and centrifuged again. The supernatant was discarded again and the cells were re-suspended in 10 ml complete growth medium, transferred to a 75cm<sup>2</sup> cell culture flask and incubated at 37°C. After 72 hours, the medium was discarded and cells were fed with complete growth medium and returned to the incubator. Cultures were examined twice per week thereafter.

## **Passaging Cell Cultures**

When cultures reached confluence, spent medium was aspirated and the cells were washed with 10 ml of Hank's Balanced Salt Solution. This process was repeated twice in order to flush away any remaining medium. A volume of 1 ml of 0.25% Trypsin/EDTA was then pipetted into the flask and the flask was incubated for 5-10 minutes until cell detachment was observed under an inverted microscope. A volume of 2 ml of complete growth medium was added to inactivate the Trypsin and the solution was pipetted to re-suspend the cells prior to freezing, counting or further experiments.



## **Cell Counting**

Haemocytometer method:

An aliquot of 50  $\mu\text{l}$  of cell suspension was pipetted into a micro-centrifuge tube. A coverslip was placed over the chambers of the haemocytometer and 20  $\mu\text{l}$  of cell suspension was slowly pipetted against the short side of the coverslip so that the suspension was drawn into the chambers. The haemocytometer was placed onto the stage of an inverted phase contrast microscope and the middle 25 squares of one chamber (centre grid, 1  $\text{mm}^2$ ) were viewed. The number of cells in these 25 squares was counted and the same process was repeated for the other chamber. The average number of cells in the centre grid was calculated and multiplied by  $10^4$  to calculate the number of cells per 1ml of suspension. This number was multiplied by the total volume of cell suspension in ml to calculate the total number of cells.

Coulter Counter Method:

The Coulter Counter was flushed using Isoton II diluent. The particle size was set to 10  $\mu\text{m}$  and a background count was performed using diluent only. A volume of 100  $\mu\text{l}$  of cell suspension was then pipetted into an Accuvette II vial and 10 ml of Isoton II diluent was added. Each suspension was counted 3 times and an average cell count was calculated from this.

## **Cell Freezing**

The cell suspension was pipetted into a centrifuge tube and centrifuged at 1500 RPM for 5 min. The supernatant was aspirated and the cells were re-suspended at a concentration of  $1 \times 10^6$  per 500  $\mu\text{l}$  complete growth medium containing 10% Foetal Calf Serum (FCS). The same volume for dimethyl sulfoxide (DMSO, cryoprotectant) with FCS was added drop wise to the cell suspension, shaking the solution gently after each drop. The suspension was then transferred into cryovials in 1 ml aliquots. The cryovials were then placed in a  $1^\circ\text{C}$  freezing container (Mr Frosty Cryo). The container was placed in a  $-70^\circ\text{C}$  freezer for 24 hours and then transferred into a liquid nitrogen refrigerator.

To revive the cells, the cryovials were placed into a 37°C water bath for 1-2 min. Cells were then pipetted aseptically into a centrifuge tube containing transport medium and centrifuged at 1500 RPM for 5 min. The supernatant was aspirated and cells were re-suspended in 1-2 ml of complete growth medium. The cells were then transferred to culture flasks containing 10 ml of complete growth medium and incubated at 37°C. Following cell attachment, medium was aspirated and replaced with fresh complete growth medium to remove any residual DMSO.

### **Fixation and Immunostaining of Cell Monolayers**

#### **A. Tris Buffered Saline (TBS)**

Distilled water  
Trizma Salt  
Sodium Chloride  
Bovine Serum Albumin

#### **B. Primary Antibodies**

Anti-AT1 receptor antibody produced in rabbit (Anti-AGTR1, Sigma Aldrich)  
Anti-AT2 receptor antibody produced in rabbit (Anti-AGTR2, Sigma Aldrich)  
Anti-Actin,  $\alpha$ -Smooth Muscle antibody produced in mouse (Sigma Aldrich A5228)

#### **C. Vectastain Universal Elite ABC Kit**

#### **E. Diaminobenzidine Solution (DAB)**

### **Procedure**

Glass coverslips were placed into a sterile Petri dish and covered with 10 ml of Complete Growth Medium. A volume of 100  $\mu$ l of cell suspension was pipetted over each coverslip and allowed to grow to 50-60% confluence.

The medium was aspirated from the Petri dish and the monolayers washed 3 times with TBS. The cells were covered with a 1:1 ratio of methanol to acetone pre-chilled to -20°C and placed rapidly into a -20°C freezer to fix for 10 minutes. The fixative was then removed from the Petri dish and the cells were allowed to air dry.

When ready for staining, the cells were carefully rehydrated with TBS. An aliquot of 40  $\mu$ l of *primary* antibody was pipetted onto each coverslip and incubated in the closed Petri dish for 30 min at room temperature. The cells were then rinsed twice with TBS. A volume of 40  $\mu$ l of *secondary* antibody was then pipetted onto each coverslip and incubated in the closed petridish for 30 min at room temperature. The cells were then rinsed twice with TBS. An aliquot of 40  $\mu$ l of streptABComplex/HRP solution was pipetted onto each coverslip and incubated in the closed petridish for 30 min at room temperature. The cells were rinsed twice with TBS. Finally, DAB solution was pipetted onto each slide and incubated for 5 min before rinsing twice with distilled water.

Cells were counterstained by pipetting Harris's haematoxylin into the petri dish for 15 seconds. The haematoxylin was then removed and the nuclei blueed by rinsing several times with warm tap water. The cells were serially dehydrated by rinsing with graded alcohol solutions. Each coverslip was dipped in Xylene and mounted in styrolite onto glass slides.

### **Fixation and Immunostaining of Frozen Sections and Paraffin Sections**

Frozen sections and paraffin sections were cut from harvested tissue, which was either collected fresh or placed in formalin. This was performed by an experienced hospital laboratory technician. Once mounted on coverslips, the immune-staining process followed the same method as outlined above.

### **Collagen Gel Contraction (Colgen 2006)**

Collagen from rat tail (Sigma Aldrich C7661)  
1M Sodium hydroxide (NaOH)  
Glacial acetic acid 99.9%  
24-well cell culture plates  
0.22  $\mu$ m syringe-driven filter units

### **Preparation of Collagen for Use in Collagen Gels**

Acetic acid solution (0.2%) was made from 99.9% glacial acetic acid and water. The solution was sterilized using the syringe-driven filter unit and cooled to 4°. Under sterile

conditions, the rat tail collagen was mixed with 0.2% acetic acid to a concentration of 6 mg/ml of collagen solution. The solution was placed on a motorised stirrer at 4°C for 2-5 days until the collagen was completely dissolved in solution. The solution was then diluted with sterilized water to a concentration of 3 mg/ml and 0.1% acetic acid. The solution was placed on a motorised stirrer at 4°C for one further day and subsequently stored at 4°C.

### **pH (NaOH) Titration of Collagen**

A volume 400 µl of DMEM was pipetted into each of 8 Eppendorf tubes. Thereafter, a volume of 0.2ml of prepared collagen solution (3 mg/ml) was added to the first Eppendorf tube and 1 µl of 1 M NaOH was pipetted into the same tube. The mixture was pipetted up and down 3 times. The resulting collagen concentration was 1 mg/ml. The process was repeated in the remaining 7 Eppendorf tubes using increasing amounts of NaOH (2-7 µl). The rigidity and colour of the gels with different volumes of NaOH were compared in order to determine the volume of NaOH that produced the most rigid gel with a neutral pH.

### **Preparing Cells Before Suspension in Collagen Gels**

A. Complete Growth Medium

B. Experimental variables

*[Val<sup>5</sup>]-Angiotensin II acetate salt hydrate* (AT1 receptor agonist - A2900 SIGMA)

*Irbesartan* (AT1 antagonist - I2286 SIGMA)

*S-(+)-PD 123177 trifluoroacetate salt hydrate* (Selective AT2 receptor antagonist - P5749 SIGMA)

*1-Methylhydantoin* (AT2 receptor agonist - M49887 ALDRICH)

*Lisinopril* (ACE Inhibitor - L2777 SIGMA)

A confluent layer of myofibroblasts was detached from the culture flask by aspirating spent medium and pipetting 1 ml of 0.25% Trypsin/EDTA solution. The cells were then centrifuged and re-suspended in complete growth medium. Cells were then counted using the Coulter Counter. The remaining suspension was centrifuged and the

supernatant removed. Fresh complete growth medium was added (+/- experimental variable) in order to reach a final concentration of  $1.5 \times 10^5$  cells/ml following the addition of the collagen solution.

### **Pouring Cell Populated Collagen Gels**

Collagen gels were prepared under sterile conditions as outlined above. A volume of 660  $\mu$ l of cell suspension (+/- experimental variable) was added to a sterile tube. A volume of 330  $\mu$ l of 3 mg/ml collagen solution was added to the tube and the appropriate volume of 1 M NaOH was also added quickly. The solution was mixed up and down with a pipette and 500  $\mu$ l of the mixture transferred to a 1.9 cm<sup>3</sup> well. The gels were left to solidify at room temperature for 20 min. An equal volume of complete growth media (+/- experimental variable) was added to each well. The gel was dissociated from its mold by gently running the tip of a 200  $\mu$ l pipette around the rim of each well. The well plate was returned to the incubator at 37°C

### **Measurement of contraction**

A digital camera (Canon Ixus 8.0 Megapixel) was used for image acquisition every six hours following solidification of cell-populated collagen gels. 6-well plates containing cell-populated collagen gels were placed on a portable lightbox and photographs were taken at standardised 40 cm above the laboratory worktop. The images were digitally uploaded using ImageJ Software Version 1.32 (downloaded from National Institutes of Health, Bethesda, MD). The surface area of each image was calculated using this software and the measured contraction relative to time presented in numerical and graphical form.

### **Measurement of intracellular free calcium $[Ca^{2+}]_i$ using a fluorescence micro plate reader**

Measurement of  $[Ca^{2+}]_i$  was performed as previously described in the literature (Robinson *et al.*, 2004). The measurement of  $[Ca^{2+}]_i$  was performed by seeding a density of 10,000 cells/well in black 96 well plates (Griner, UK) in 100  $\mu$ l of growth medium. At sub confluence, the cell culture medium was then replaced with FBS free medium for 24

hr in order to synchronize cells into a non-proliferation stage. The cell were then loaded with 20 µl 25 µM Fura-2 acetoxymethyl ester (AM) in cell medium at 37°C for 30 min followed by 30 min at temperature to minimize dye leakage and sequestration into intracellular organelles. After loading, the cells were washed 2-3 times with 200 µl of Hank's Buffered Salt Solution (HBSS) to remove excess fluorescent dye. The cells were then treated with angiotensin x min in a calcium free medium. The cells were washed twice with 200 µl HBSS and then a volume of 100 µl HBSS /wells was added to each well. The same procedure was carried out for the untreated cells. The intensity of the fluorescence's of Fura-2 AM was measured at emission wavelengths of 340 nm. The relative changes in calcium concentration using Fura-2 AM were determined by calculations of

$$[[Ca^{2+}]_i = K_d \beta (f - f_{min}) / (f_{max} - f).$$

$K_d$ , the Ca-Fura-2 dissociation constant,  $\beta$  the fluorescence intensity ratio, excited at 380 nm without and with Ca;  $f_{min}$  and  $f_{max}$  (Robinson *et al.*, 2004). All values were expressed as ratio units of the Fura-2 AM fluorescent intensity (Robinson 2004).

Note that the above experiments were only carried out once and they were unsuccessful.

### **Statistical Analysis**

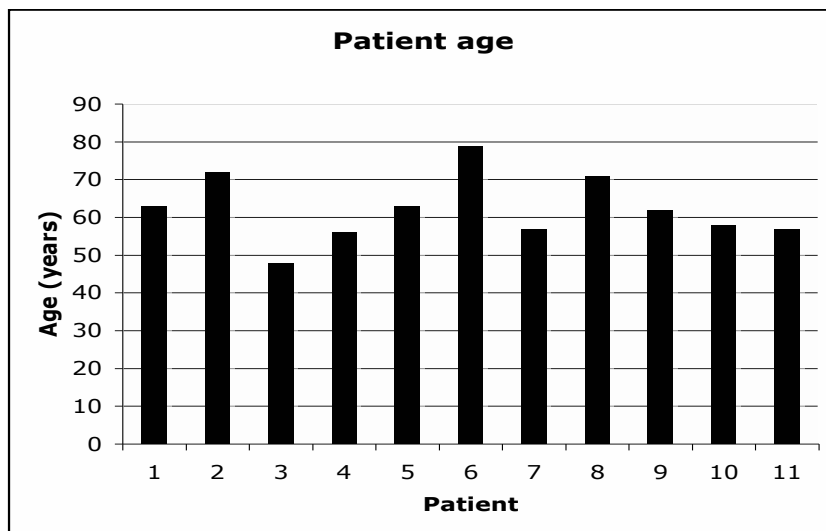
All control and test data collected from the different experiments were analysed using Statistical Package for Social Sciences (SPSS) version 17, Student's – t test and ANOVA test. Data obtained were expressed as mean  $\pm$  standard deviation (S.D) where possible. Each experiment was repeated for 3-6 times where possible. A value of ( $p < 0.05$ ) was taken as significant.

## **Chapter 3**

### **Results**

## Patients and Epidemiology

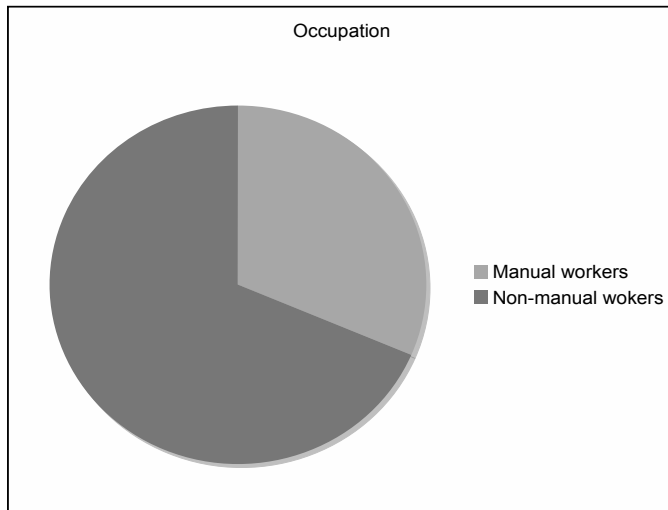
All patients were recruited from the Department of Plastic Surgery at Royal Preston Hospital. This study recruited 11 patients who had attended for surgical treatment of Dupuytren's. All patients gave written consent to be included in the study. The indication for surgery for all patients was that the disease was restricting their hand function. The degree of restricted function was varied, but in all cases there was a significant deficiency in their ability to perform at least one activity of daily living. As such, these patients had to undergo surgery. The results presented in this thesis are based on 11 patients (9 males, 2 females) between the ages of 48 to 79 (mean=62.4) who underwent surgery for Dupuytren's disease at Royal Preston Hospital during the period of the study and who consented to their tissues being used for research. Figure 3.1 shows the age distribution of the 11 patients who were included in this study. The results show that there is a relatively wide variation in age, but that all patients were middle age or older.



**Figure 3.1:** Bar chart showing age of patients

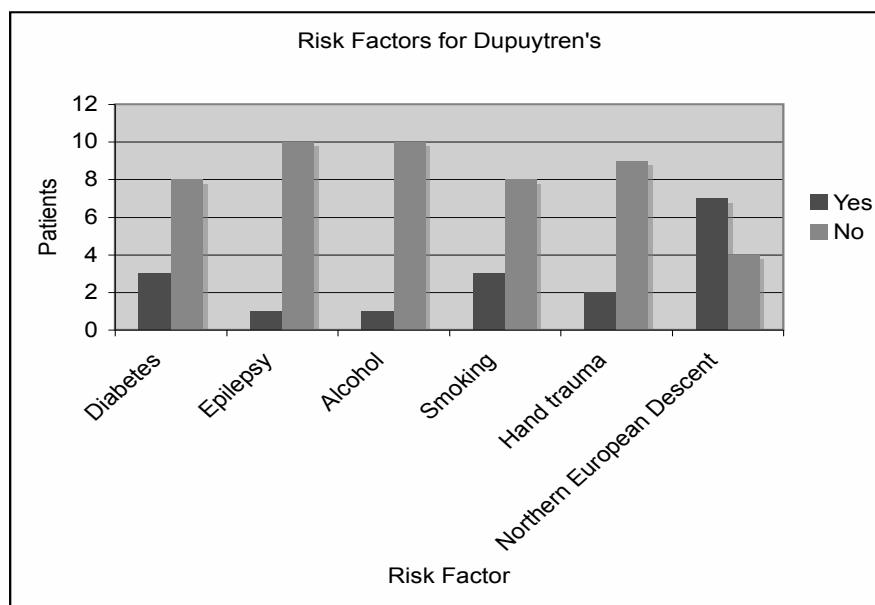
Figure 3.2 (below) shows the proportion of manual workers to non-manual workers in the 11 patients. The patients' occupations varied greatly, but 5 of out 11 patients (45%) were manual workers including builders and mechanics. The remaining 55% of the patients had non-manual jobs including teachers, solicitors and other professionals. Please note that these figures are referring to the main occupation of the patients until the time of retirement, as several of the patients were retired at the time of the study.





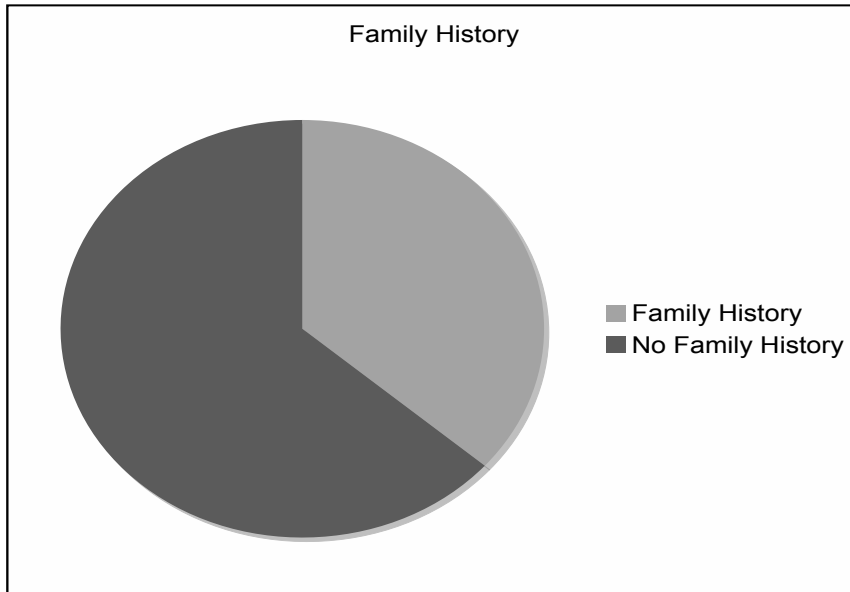
**Figure 3.2:** Pie chart showing the percentage of manual non-manual workers in this study

Dupuytren's disease is associated with a number of risk factors. The results presented in Figure 3.3 show that all patients exhibited at least one risk factor or condition associated with Dupuytren's disease. The most common of these was a Northern European background. The results presented below also show that certain patients in this study also suffered with diabetes, epilepsy and alcohol abuse. Furthermore, 3 patients were smokers and 2 patients had suffered previous hand trauma.



**Figure 3.3:** Bar chart showing patients' risk factors for Dupuytren's disease

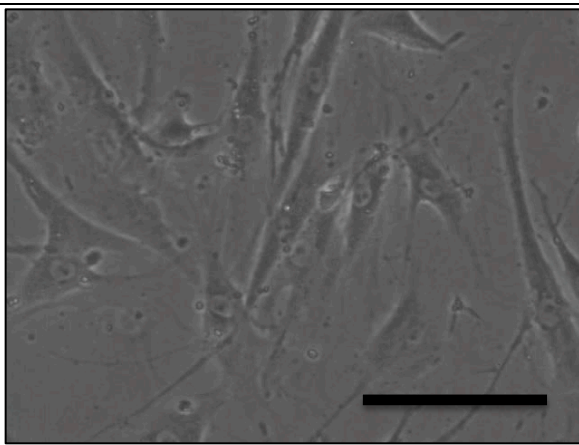
Figure 3.4 shows the proportion of patients who had a positive family history of Dupuytren's disease among the 11 patients employed in this study. The results show that of 4 out of 11 patients (36.4%) had a positive family history of Dupuytren's disease.



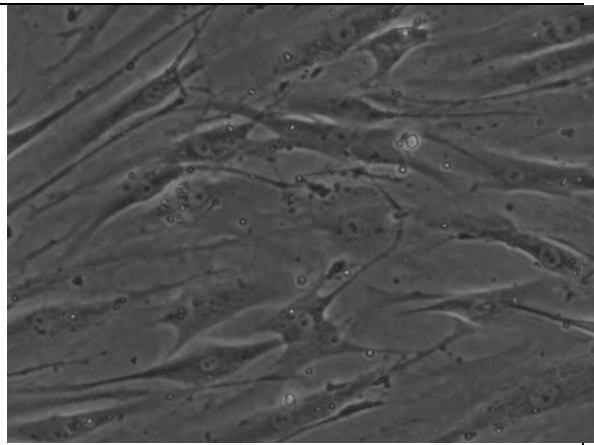
**Figure 3.4:** Pie chart showing family history of Dupuytren's disease.

## Myofibroblast Cell Culture

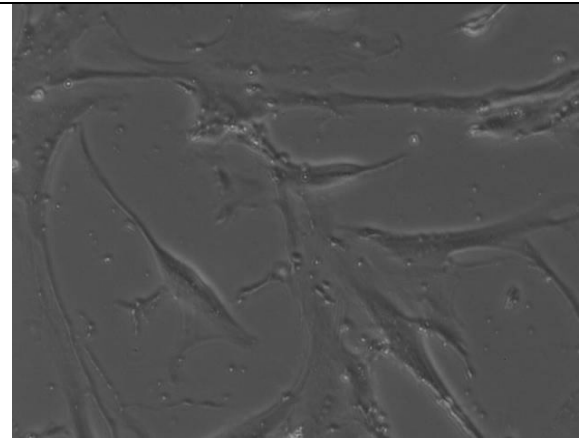
Tissues were harvested from all 11 patients over a 4-month period. They were dissociated using collagenase and cultured for 4-5 months. Figures 3.5-3.14 show the cell specimens from patients 1-10. The results presented in these figures show that these are large, spindle-shaped cells with nuclear indentations and a branching structure with numerous long cytoplasmic connections. Note that these cells can resemble normal fibroblasts, but that immunostaining for  $\alpha$ -SMA was subsequently performed to confirm that the cells were in fact myofibroblasts.



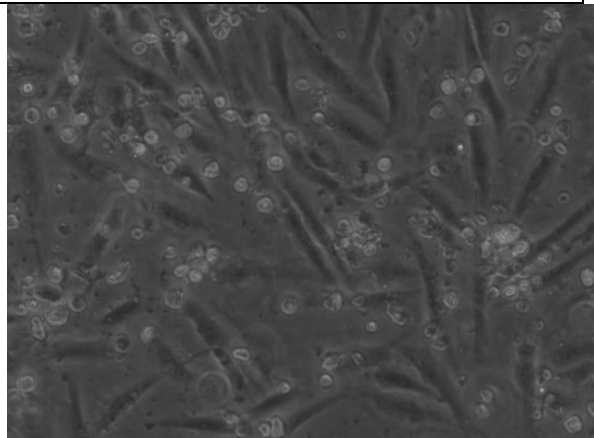
**Figure 3.5:** Specimen 1  
(scale bar 200  $\mu$ m, 100x magnification)



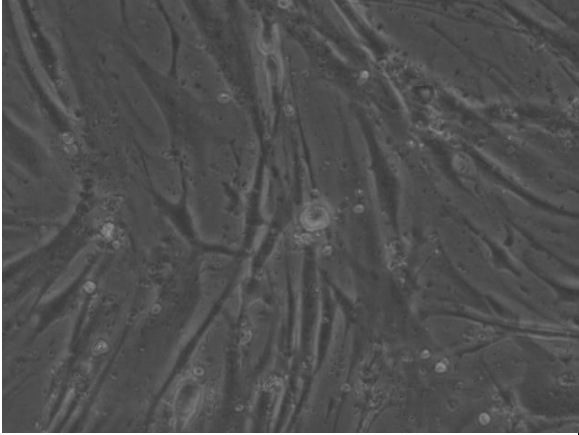
**Figure 3.6:** Specimen 2



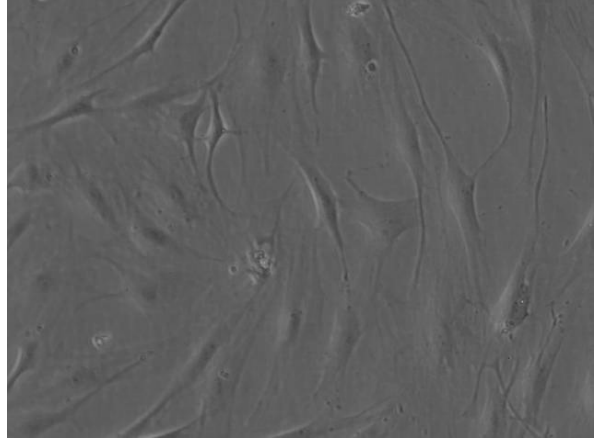
**Figure 3.7:** Specimen 3



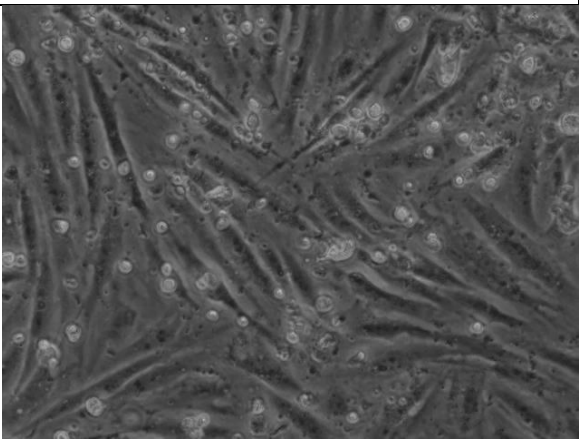
**Figure 3.8:** Specimen 4



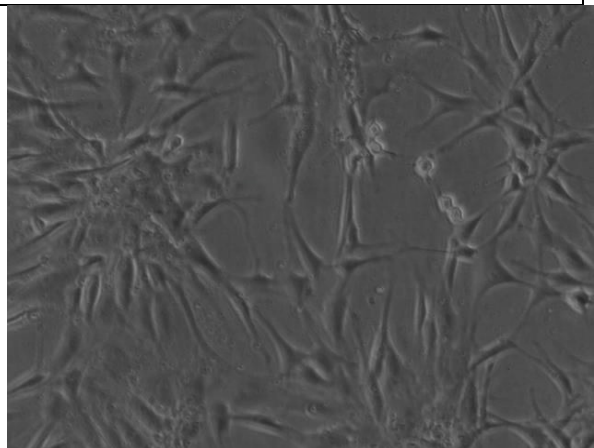
**Figure 3.9:** Specimen 5



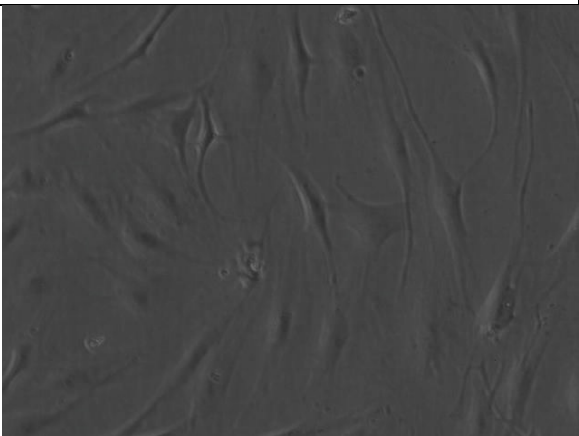
**Figure 3.10:** Specimen 6



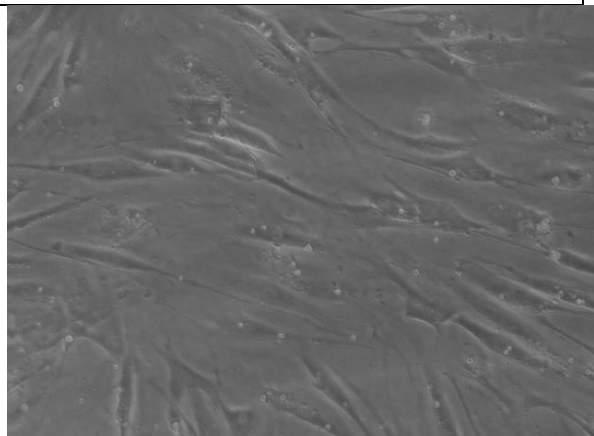
**Figure 3.11:** Specimen 7



**Figure 3.12:** Specimen 8



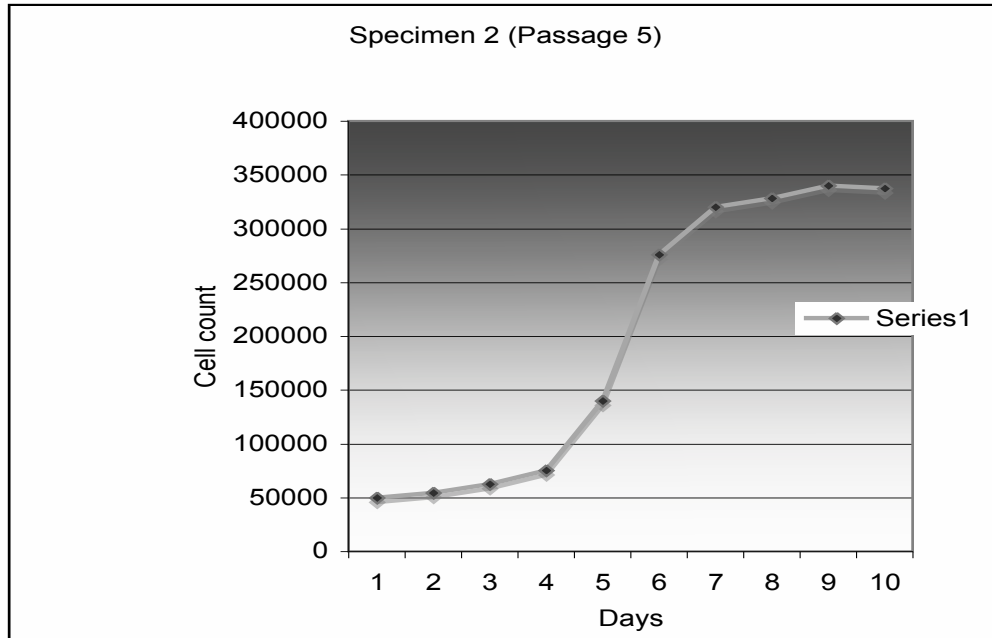
**Figure 3.13:** Specimen 9



**Figure 3.14:** Specimen 10

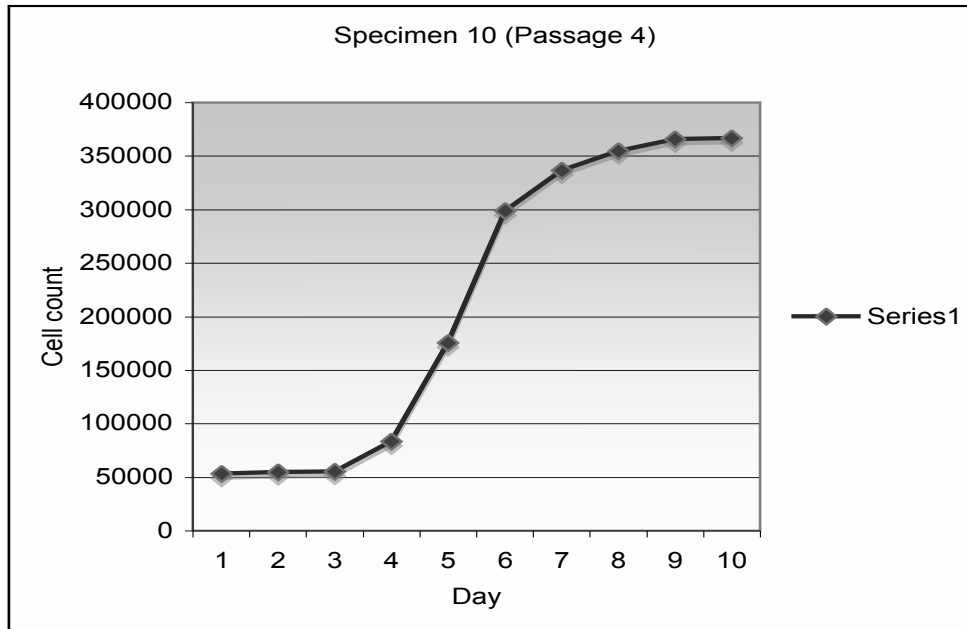
## Growth curves

Figure 3.15 shows data of a typical growth curve for myofibroblasts obtained from patient number 2. The results show that these myofibroblasts can grow in a time-dependant manner from day 1 to day 10. There was slow growth from day 1 to day 4, followed by a rapid growth from day 5 to day 7 and then reaching an almost plateau level between day 7 and day 10.



**Figure 3.15:** Typical growth curve for Dupuytren's myofibroblasts (specimen 2, passage 5) in 25cm<sup>3</sup> culture flasks over a period of 10 days.

Figure 3.16 shows the growth curve for myofibroblasts obtained from patient number 10. Again, that data show a slow growth for day 1 to day 4, following by a rapid growth for day 5 to day 7, reaching maximum growth within days 8-10.



**Figure 3.16:** Typical growth curve for Dupuytren's myofibroblasts (specimen 10, passage 4) in 25cm<sup>3</sup> culture flasks over a period of 10 days.

## Immunostaining

Figures 3.17-3.23 show typical micrographs of immunostaining for AT1, AT2 and  $\alpha$ -SMA. This was performed on cell monolayers, paraffin sections and also on frozen sections.

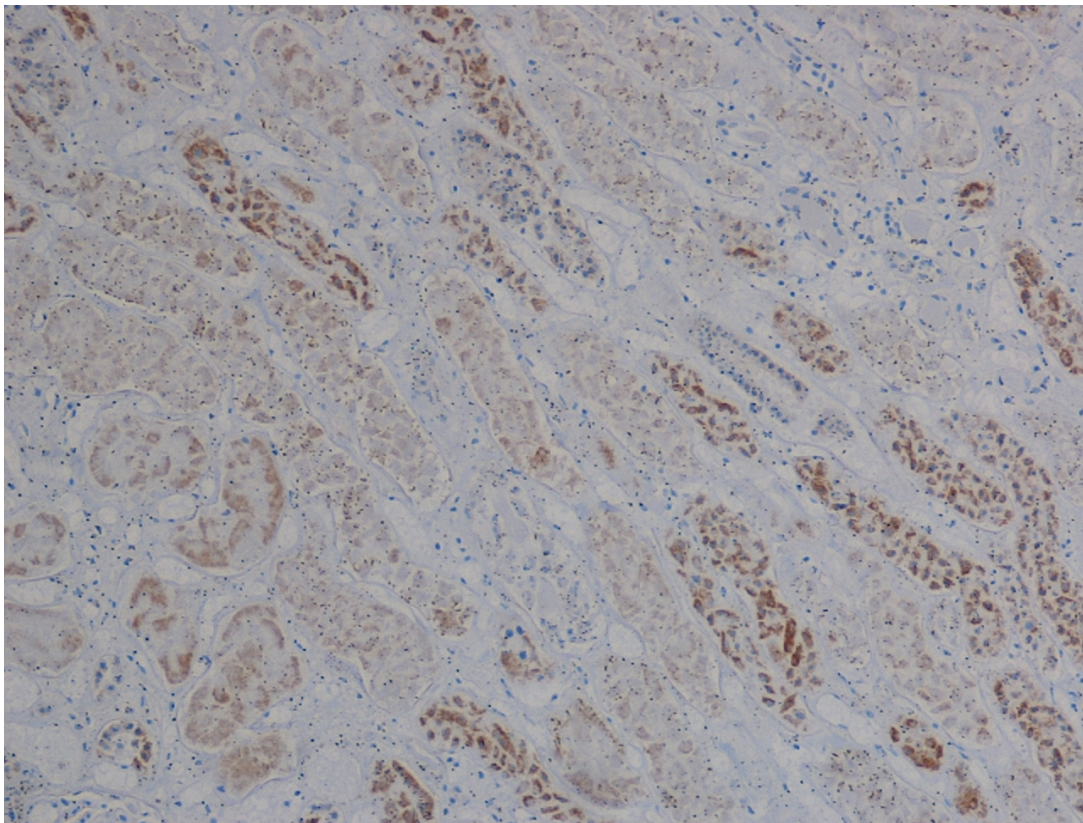
### Cell monolayers

All cell monolayers were negative for the antibodies tested. This was tested using varying concentrations of primary antibody as well as incubation periods ranging from 30 min to 24 hours.

### **Paraffin sections**

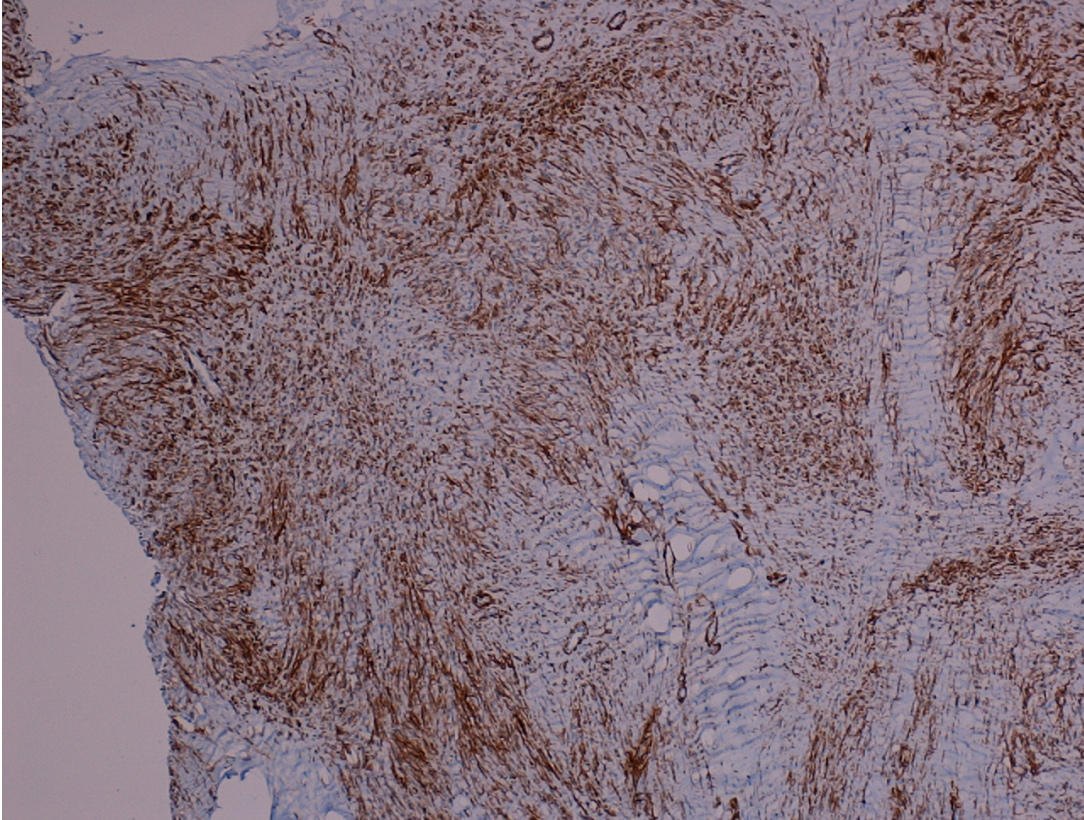
Using paraffin sections, all specimens showed positive staining for  $\alpha$ -SMA, as well as the kidney control section used (Figure 3.17, 3.18). However all specimens, on paraffin section, stained negatively for AT1 and AT2 receptors (Figure 3.19, 3.20). This was tested using varying concentrations of primary antibody as well as incubation periods ranging from 30 min to 24 hours.

Using frozen sections, only 1 out of 5 specimens stained mildly positively (specimen 6) for AT1 receptors whilst the remainder of specimens stained negatively for AT1 receptors (Figure 3.21, 3.22). However, all frozen sections stained positively for AT2 receptors (Figure 3.23).



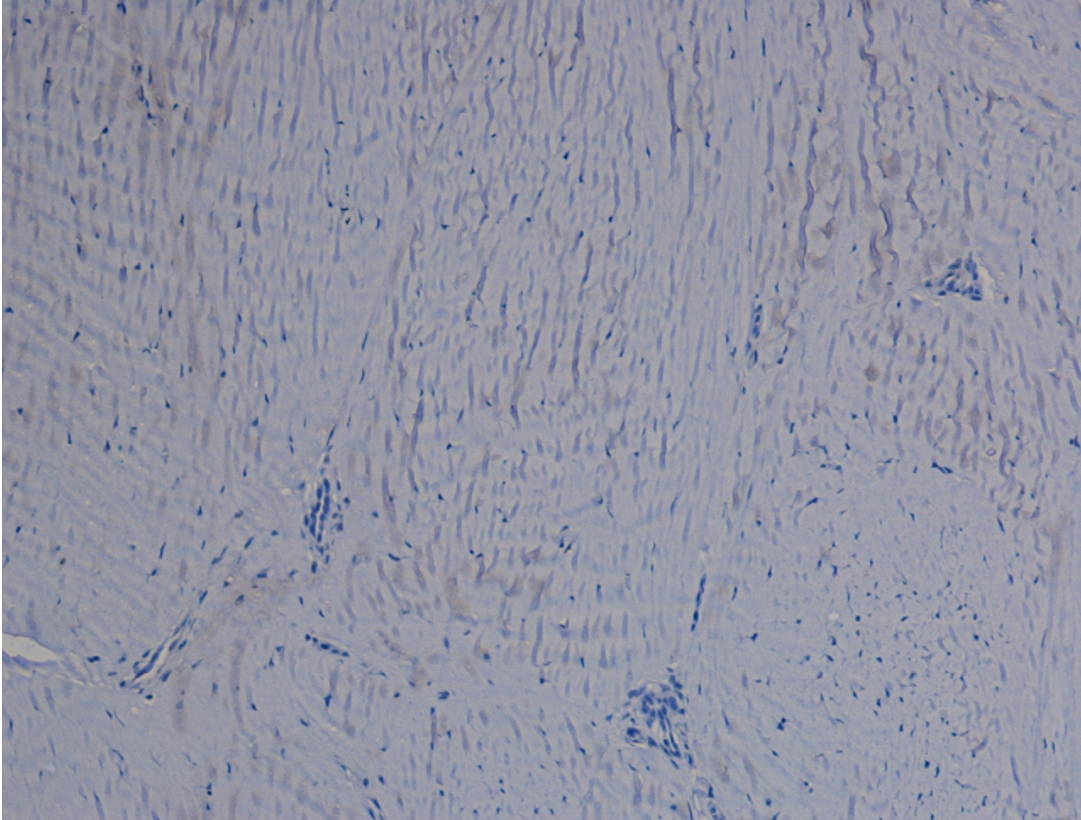
**Figure 3.17:** Micrograph showing kidney paraffin section stained for alpha smooth muscle actin ( $\alpha$ -SMA). This micrograph is typical of 3 different experiments on similar tissues. 20x magnification.



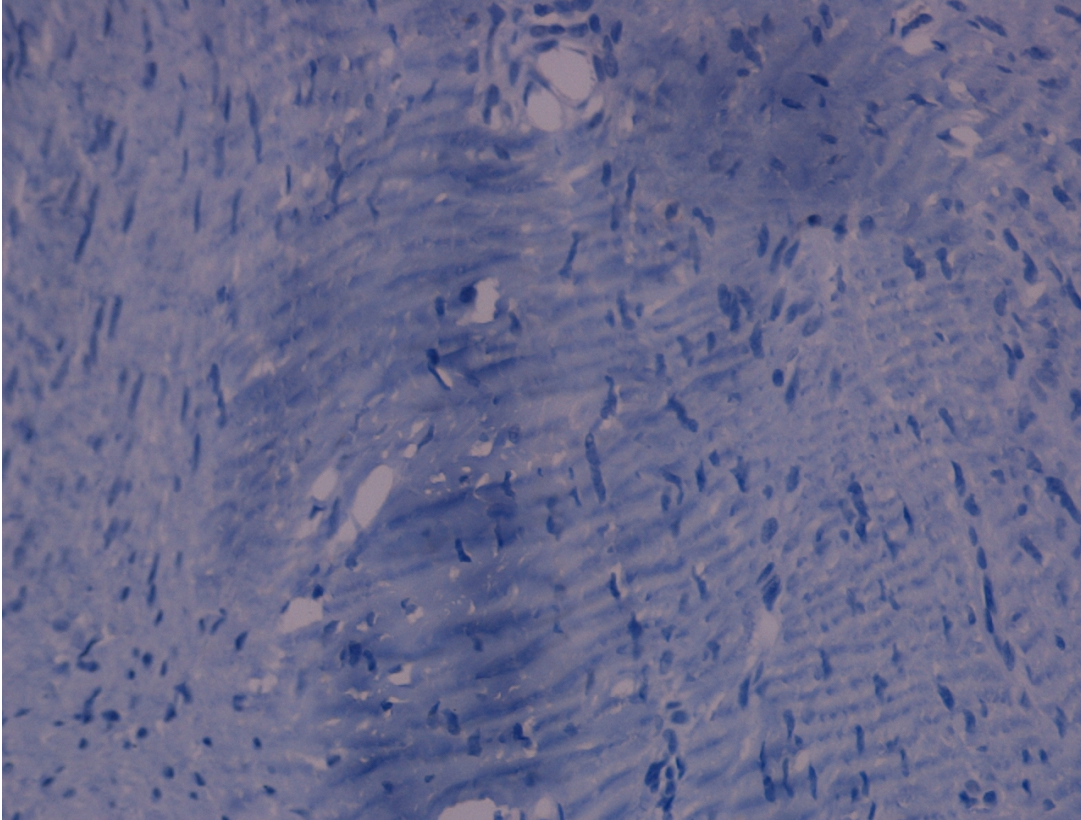


**Figure 3.18:** Micrograph showing Dupuytren's specimen 6, paraffin section stained for  $\alpha$ -SMA. This is typical of 6 such different experiments on different specimens. 10x magnification.





**Figure 3.19:** Micrograph showing a paraffin section stained for AT1 receptors in myofibroblasts from patient number 5. This is a negative result and it is typical of 20 such different experiments at different primary antibody concentrations as well as different incubation periods. 10x magnification.

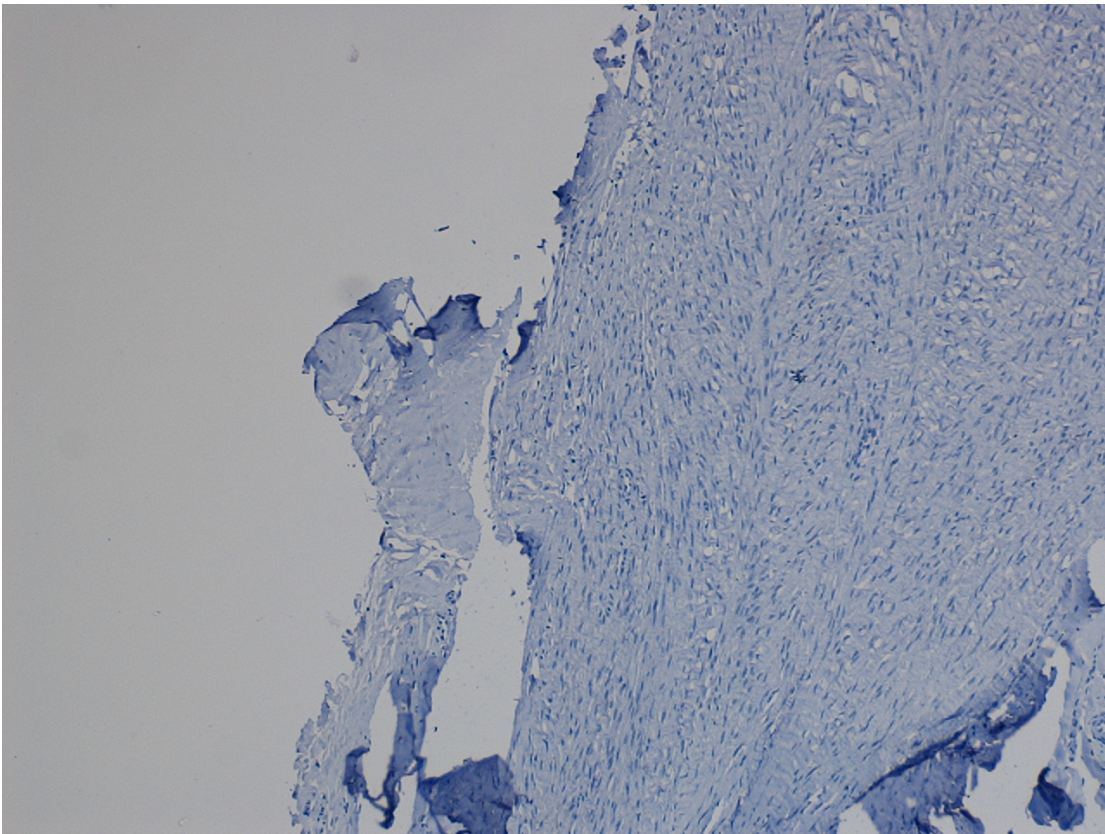


**Figure 3.20:** Micrograph showing a paraffin section stained for AT2 receptors in myofibroblasts from patient number 5. This is a negative result and it is typical of 20 such different experiments at different primary antibody concentrations as well as different incubation periods. 10x magnification.

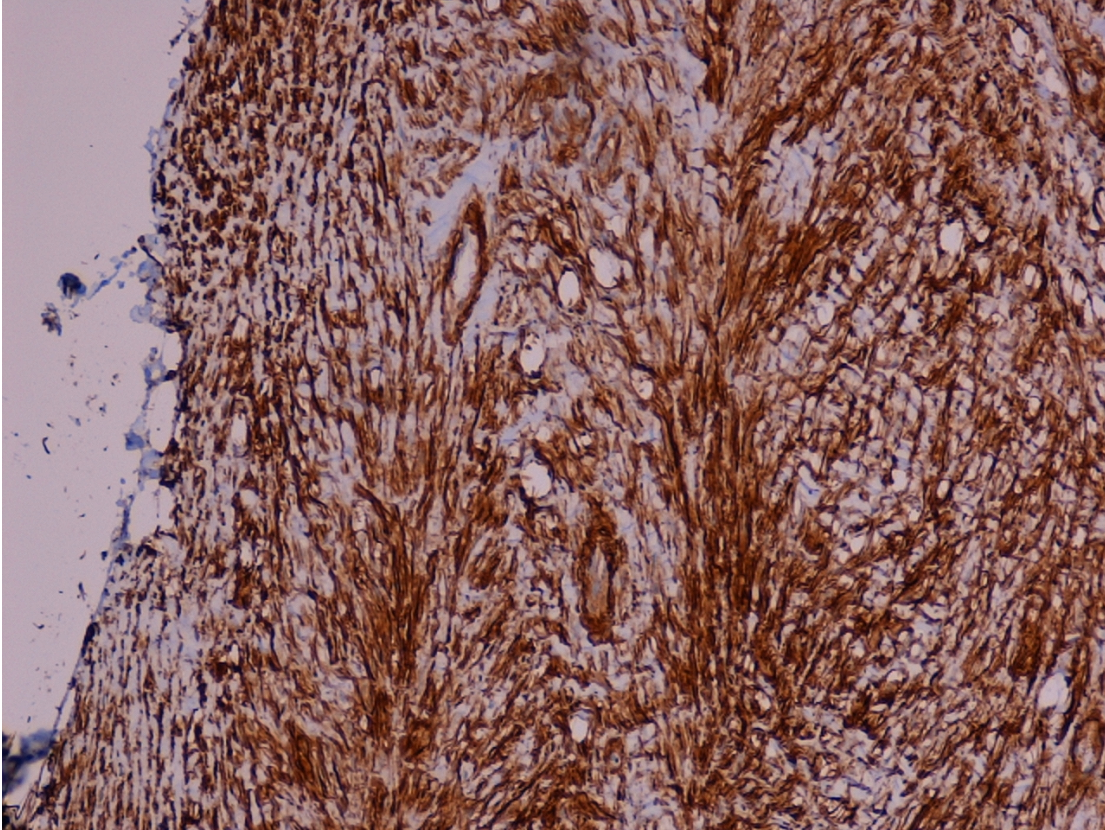


**Figure 3.21:** Micrograph showing a frozen section stained for AT1 receptors in myofibroblasts from Dupuytren's patient number 6. This is a mildly positive result. Note that 12 such different experiments at different primary antibody concentrations as well as different incubation periods were carried out on other specimens, all of which were negative for AT1 receptors. 6x magnification.





**Figure 3.22:** Micrograph showing a paraffin section stained for AT1 receptors in myofibroblasts from patient number 6. This is a negative result and it is typical of 12 such different experiments at different primary antibody concentrations as well as different incubation periods. 4x magnification.



**Figure 3.23:** A typical micrograph showing a frozen section obtained from Dupuytren's specimen 6, stained for AT2 receptors. This is a positive result and extensive staining can be observed throughout the tissue section. This is typical of 9 such different experiments. 20x magnification.

## **Chapter 4**

### **Discussion**

## Discussion

This study was designed to investigate the condition of Dupuytren's disease and this discussion will focus on the disease itself, its aetiology, causes, diagnosis and treatment. It will also describe the role of angiotensin in the development of the disease, the isolation of the tissues and myofibroblasts, growth of the myofibroblasts using tissue culture techniques, and the identification of angiotensin receptors in the myofibroblasts. In addition, the discussion will also touch on the problems and issues involved in this type of study.

Dupuytren's disease is a common condition affecting 4-6% of Caucasians worldwide (Hurst 2009). The severity of the disease is variable, but it is progressive, and can often lead to extreme restrictions in the function of the hand of affected patients. The disease is thought to be multifactorial, but the causative factors are not yet fully understood. Dupuytren's disease has been associated with several conditions such as diabetes mellitus, alcohol abuse, epilepsy, smoking, manual labour and vibration injury (Shaw Jr, 2007). There is also a genetic component, and the highest prevalence is found on men of European descent (Ross, 1999). The genetics of the disease are not fully understood and the mode of inheritance is yet to be established. Also several studies have shown a strong familial link, many cases appear sporadically (Skoog, 1948; Burge, 1999; Ling, 1963).

Since the description of the disease by Dupuytren in 1831, the macroscopic pathology has been understood to involve contraction, or shortening of the palmar fascia (Shaw Jr, 2007). Experiments conducted in the 1950s suggested that cells were responsible for tissue contracture (Tomasek, 1999). The cells responsible were later shown to be the myofibroblasts (Gabbiani, 1992). The morphological features of the myofibroblast are a combination of those of smooth muscle cells and fibroblasts, and it is thought that these cells can create contractile force, thus propagating the contraction of the palmar fascia in Dupuytren's disease. The feature that distinguishes myofibroblasts from smooth muscle cells and fibroblasts is the presence of bundles of actin microfilaments arranged along the long axis of the cell (Tomasek, 1999). Specifically, these microfilaments contain  $\alpha$ -smooth muscle actin, which we have successfully identified in this study (Skalli, 1989).

Surgical intervention is usually considered and it is based on the degree of contraction across the metacarpophalangeal and proximal interphalangeal joints. In general, more than 30 degrees of flexion in the former or any flexion deformity of the latter would warrant intervention. The disease is progressive and therefore surgery will not cure the affected patients. Surgery is therefore offered to patients with contractures that cause a significant loss of function or difficulty with self-care. Earlier operative treatment is considered in certain circumstances, such as in patients with rapidly progressive disease. Surgery is relatively contraindicated in patients with very minimal restriction of function or in those whose major concern is cosmetic rather than functional. Surgery is not offered to patients with a general medical condition that places them at unreasonable risk of severe complications. There are several surgical methods of resecting the diseased tissue, a detailed description of which is beyond the scope of this discussion. However, all surgical options carry similar risks, including bleeding, nerve damage, artery damage, necrosis of skin, failure to correct the deformity and complex regional pain syndrome (Janis, 2007). Although many non-surgical treatments have been previously employed, such as radiotherapy, ultrasound, interferons and steroids, none have been proven to have consistent success (Janis 2007). One of the most encouraging developments in non-surgical treatment of Dupuytren's disease is collagenase enzymatic fasciotomy, in which the enzyme collagenase is injected into diseased tissue causing lysis of diseased cords. However, structures such as tendons and nerves, which lie in close proximity and are also composed of type I collagen, are also susceptible to injury from collagenase (Shaw Jr, 2007). It is therefore technically challenging to ensure that normal structures are not damaged with this therapy, and clearly a more selective therapy would be favourable.

Thus, the treatment of Dupuytren's disease is fraught with difficulties. All treatment modalities have subject to a high risk of disease recurrence (Shaw Jr, 2007). This reflects the fact that the disease pathology on a cellular level is not fully understood. It is therefore important to investigate this debilitating condition in further detail in order to develop a potential therapy. As described, the myofibroblast is the key cell involved in Dupuytren's disease. Chronic fibroblast activation leads to excessive collagen synthesis and contraction. A full appreciation of the cellular mechanisms which activate the myofibroblast to function in this manner is required in order to develop a therapy that can specifically target the myofibroblast and reduce its fibrotic and contractile activity.



In this thesis, the role of Angiotensin II in relation to Dupuytren's disease has been investigated. The Renin-Angiotensin (RAS) has long been understood to play a circulating hormonal role in fluid and electrolyte balance and blood pressure control (Johnston, 1990). However, there is much evidence showing ANG II to play a local role in wound healing, fibrous tissue formation and regulation of the extracellular matrix, particular in cardiac and renal models (Sun, 1997a; Weber, 1997; Sun 1996; Sun, 2000; Sun, 1993; Sun, 1997). It is thought that ANG II is generated at the site of injury. For example, ANG II concentration is greater in scar tissue following myocardial infarction compared to normal myocardium (Yamagishi, 1993). ANG II has also been implicated in renal tubule-interstitial fibrosis associated with ureteric obstruction (Klahr, 1995; Weber, 1997). Numerous studies have shown the requisite components of the RAS, such as renin, angiotensin and ACE, are present in tissues such as the brain, heart and kidney (Dinh, 2001). In the rat heart and kidney, it has also been shown that ACE and Angt II binding was of high density in areas of fibrosis compared to non-injured areas (Weber, 1997).

Studies of fibrosis in kidney (Mizuno, 1998) and lung fibroblasts (Nguyen, 1994) have shown a reduction in the population of fibroblasts and myofibroblasts in culture when treated with ACE inhibitors. The use of ACE inhibitors as an anti-fibrotic agent in patients with renal and cardiac disease is now widely established in clinical practice. However, the role of ANG II in the skin and subcutaneous tissue has been investigated in less detail.

Acceleration in dermal tissue healing with the application of ANG II has been demonstrated in the rat excisional wound model (Rodgers, 1997). Furthermore, the ACE inhibitor Lisinopril has been shown to inhibit the rate of wound contraction and collagen deposition in the rat model (Mckirdy, 2001).

The presence of Angiotensin receptors in Dupuytren's disease has been previously investigated, revealing AT1 receptors in myofibroblasts cultured from tissues excised from 5 different patients with Dupuytren's disease (Mckirdy, 2001), which was a strong basis for the methods employed in this study.

In the present study, tissues were harvested from 11 patients undergoing surgery for Dupuytren's disease in the Department of Plastic Surgery at Royal Preston Hospital. These tissues were treated with collagenase to aid the dissociation into cells, which were successfully cultured. The growth curves obtained in the present study are comparable to those of fibroblasts as published previously (Takashima, 2001). The cells were cultured for a total period of 6-8 months, although some cell lines became susceptible to infection, particularly in the early stages of the study when this technique was initially being learnt.

Morphologically, the myofibroblasts can resemble fibroblasts in that they are large, spindle-shaped, branching cells. There are certain features that can differentiate myofibroblasts from fibroblasts, such as irregular, stellate cellular outlines with numerous long cytoplasmic connections connected by intermediate or adherence junctions. Moreover, they are connected to the extracellular matrix by cell-to-stroma attachment sites through fibronexus. They contain bundles of cytoplasmic filaments arranged parallel to the long axis of the cell, a well-developed rough endoplasmic reticulum, golgi and indented nucleus with prominent nucleoli (Ravikanth, 2011). Many of these features can be observed with electron microscopy. However, a striking histological feature differentiating myofibroblasts from fibroblasts is the presence of bundles of actin microfilaments, and more specifically, the expression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) (Tomasek 1999). For this reason, the tissues harvested in this study were tested for the presence of  $\alpha$ -SMA using the Immunostaining technique described. The results show that the tissues were strongly positive for  $\alpha$ -SMA, confirming that the cells present were myofibroblasts.

In contrast to the work conducted by McKirdy et al (2001), which showed Dupuytren's tissue to stain strongly positive for AT1 receptors, the present study has found that the tissues stained only mildly positively for AT1 receptors at best, with some specimens showing no signs of AT1 receptors. Exhaustive attempts were made to establish the presence of AT1 receptors, including numerous concentrations of primary antibody and various incubation times. It is noteworthy that this study was larger in that tissues from a larger number of patients were used.

In addition, this study has shown that tissues obtained from the eleven patients who had Dupuytren's disease stained positively in an extensive manner for AT2 receptors in all patients. This positive staining was far more readily observed than that for AT1 receptors on the first attempts, without the need to modify the incubation periods or concentrations of primary antibody. This observation suggests that the expression of AT2 receptors is more prominent than the expression of AT1 receptors in Dupuytren's disease. This is a new discovery, which has not been found on other studies.

Although both AT1 and AT2 receptors have been identified in a variety of organs, much less is known about the role of AT2 receptors compared to AT1 receptors. It is apparent that AT1 receptors are responsible for mediating all the well-known stimulatory actions of ANG II on blood pressure, electrolyte homeostasis (Timmermans, 1999). AT2 receptors have been identified in several tissues including the brain, adrenal gland and heart (Allen 2000), and are more widely distributed in fetal tissues than in adults (Brunton, 2005). It is now known that AT2 receptors mediate anti-growth and apoptotic actions of ANG II, and there is a growing body of evidence that the stimulation of AT2 receptors may offset or oppose the AT1 receptor-mediated actions of ANG II on cell growth (Gallinat, 2000). In the brain, the administration of an AT2 receptor antagonist has been shown to potentiate the stimulatory effects of ANG II on drinking behavior and vasopressin secretion, demonstrating an opposing effect of AT2 receptor stimulation to AT1 (Höhle, 1996). In chronic heart failure, AT2 up-regulation occurs in fibroblasts and is associated with down-regulation of AT1 receptors (Gallinat, 2000). Opposing actions of AT1 and AT2 receptors have also been demonstrated in the kidney. Furthermore, AT2 receptor expression in the heart and it has been shown to increase substantially following myocardial infarction (Gallinat, 2000). It therefore seems apparent that AT2 receptors play a significant role in tissue repair in a variety of organs (Gallinat 2000). Importantly, the presence of AT2 receptors has been identified in the skin (Viswanathan 1992). Furthermore, an experimental wound healing model in the skin of young rats has shown that ANG II receptor expression and this is enhanced during experimental wound healing, and that the major proportion of this increase was due to AT2 receptors (Viswanathan, 1992).

One of the main aims of this study was to investigate the effects of ACE inhibitors in Dupuytren's disease. This was based on the previous discovery of the presence of AT1

receptors in these tissues. It was hypothesized that ACE inhibitors would exhibit anti-fibrotic effects via blockade of AT1 receptors. The identification of AT2 receptors in this study leads a new direction because of the existing evidence of opposing actions of AT1 and AT2 receptors as described above. As discussed, the role of AT2 receptors is much less understood than AT1 receptors. It would therefore be appropriate to investigate not only the effects of ACE inhibitors and AT1 receptor antagonists, but also the effects of AT2 agonists and antagonists in Dupuytren's disease.

Numerous attempts were made in this study to generate a three-dimensional collagen gel model in order to measure the effect of various agents on the contraction of myofibroblasts. Whilst a clear protocol was followed precisely, the method could not be carried out successfully because the collagen gel did not solidify following the addition of the cell suspension, despite the fact that the addition of NaOH was titrated to the nearest  $\mu\text{l}$ . Therefore, this study attempted to modify the protocol with reference to other collagen gel contraction studies. However, none of these actions resulted in the ability to produce a cell-populated collagen gel that would solidify in order to enable measurements of contraction. This part of the study had to be abandoned due to time restriction. However, in future studies, it may be preferable to employ a commercially available gel contraction assay kit.

### **Concluding remarks**

Together, this study has shown that it is possible to obtain primary tissues from patients with Dupuytren's disease and culture the tissues successfully into myofibroblasts over time. It is also possible to stain for the AT1 and the AT2 receptors on the myofibroblast plasma membrane. Unfortunately, because of difficulties it was not possible to measure contraction and intracellular free calcium concentrations in the myofibroblast in the absence and presence of angiotensin and its receptor antagonists. Nevertheless, the study has led to the discovery of AT2 receptors in Dupuytren's disease, which can be the basis of further studies. The study has also led to the development, understanding and learning new physiological techniques, which are required for the MSc by Research.

## **Chapter 5**

## **Conclusion**

## Conclusion

This study employed primary tissues isolated from the hands of eleven patients who suffered with Dupuytren's disease, which is caused by both genetic and environmental factors. There is some evidence that Angiotensin II (ANGT II) may be associated with this disorder and that blockers of ANGT II may help in treating this disease. This study was designed to investigate the presence of ANGT II receptors 1 (AT1) and 2 (AT2) in myofibroblasts derived from primary tissue of 11 patients who had the disease. The tissue from each patient was dissociated into myofibroblasts, employing the enzyme collagenase. Myofibroblasts from each patient was successfully cultured over a period of 8-10 days to produce normal growth curves. Following culture, myofibroblasts were studied morphologically and they were subsequently stained and treated with specific antibodies for the identification of AT1 and AT2 type angiotensin receptors. The results show the distribution of both AT1 and AT2 receptors. The results suggest that more AT2 receptors were observed in the cells compared to AT1 receptors. However, the relative abundance of these receptors cannot be verified by immunohistochemistry because there may be a difference in affinities of the respective primary polyclonal antibodies employed. Numerous attempts were made to measure both contraction and intracellular free calcium concentration in the myofibroblasts but without any successful results. The present results clearly show that it is possible to harvest myofibroblasts from tissues of patients who have Dupuytren's disease and that these myofibroblasts can be used successfully to identify AT1 and AT2 receptors. With time, it may be possible to study the process of excitation-contraction coupling in the myofibroblasts.

## Scope for Future Studies

Future studies may involve the following:

1. It is possible to measure contraction in the myofibroblasts using different physiological methods in the literature. A video edge system in conjunction with three-dimensional collagen gel can be used to measure contraction. The cells can either be electrically stimulated, or different drugs and their respective antagonists can be employed, particularly the specific agonists and antagonists of both AT1 and AT2 receptors, which have been observed in the present study. The contractile response to these agents may give information relating to the clinical response of myofibroblasts in Dupuytren's tissue in vivo. Extracellular calcium can also be varied in some experiments to determine the calcium dependency of contraction.
2. It is possible to investigate the role of cytosolic calcium in the process of contraction in the myofibroblast. Cells will be loaded with Fura 2 and intracellular free calcium concentrations  $[Ca^{2+}]_i$  measured using a micro-spectrofluorometer. The myofibroblast can be stimulated electrically or with drugs, hormones and angiotensin in the absence and presence of their respective antagonists.
3. In addition to measuring physical contractility, it may be possible to investigate the levels of such cations as sodium, potassium, magnesium, calcium, zinc, copper, iron and selenium in the presence and absence of angiotensin stimulation. Both flame photometry and ICPMS can be used to measure the ions.
4. It is also possible to study the morphology of the primary tissues and the myofibroblasts using phase contrast and electro-microscopy.
5. It is also possible to employ binding studies to identify angiotensin receptors in the tissues and cells using radio-labelled angiotensin, which may give more information relating to the relative abundance of AT1 and AT2 receptors compared to the immunohistochemistry methods used in the present study.

## **Appendix**



## **THE EFFECTS OF ACE-INHIBITORS (ANTI-FIBROTIC DRUGS) ON DUPUYTREN'S DISEASE.**

You are being invited to take part in a research study. Before you make a decision on whether you would like to participate it is important for you to understand why the research is being done and what it will involve.

**Please take time to read the following information carefully** and discuss it with others if you wish. Also please ask us or your clinician if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. Keep this information sheet for future reference. Thank you for reading.

### **What is the purpose of the study?**

Dupuytren's disease is extremely difficult to either prevent or resolve. Treatment of Dupuytren's disease usually requires surgical removal as there are currently no oral treatments for this condition.

Angiotensin-Converting Enzyme (ACE)-Inhibitors are drugs commonly used in renal and cardiovascular disease to reduce scarring and fibrosis. Our study aims to determine whether ACE-inhibitors can also be beneficial to reduce scarring and fibrosis in Dupuytren's disease.

We are asking for patients to donate scar tissue from their routine surgery for removal of Dupuytren's disease for research to test the effects if ACE-inhibitors on the scarring the process. **NO** extra tissue will be taken for the study, only tissue which would be removed as part of your routine surgery would be used. **NO** tissue will be stored and any tissue not used will be disposed of according to hospital policies. The study may provide the basis for a safe therapy for patients with Dupuytren's disease.

### **Do I have to take part?**

It is entirely your decision whether or not to participate. If you do decide to take part you will be given this information sheet to keep and will be asked to sign a consent form (you will also be given a copy of the signed consent form to keep). You are still free to withdraw at any time without giving a reason. A decision not to take part or a decision to withdraw at any time will not affect the standard of care you receive.

### **What will happen to me if I take part?**

Taking part in the study will not alter your routine surgery in any way. You **WILL NOT** be required to have any extra hospital visits or have any extra investigations. The study only involves tests on the samples being donated.

**What is being tested?**

Angiotensin-Converting Enzyme (ACE)-Inhibitors such as *Enalapril* are drugs commonly used in renal and cardiovascular disease to reduce scarring and fibrosis. Our study aims to determine whether ACE-inhibitors can also be beneficial to reduce scarring and fibrosis in Dupuytren's disease.

**What are the disadvantages or risks associated with taking part?**

There are NO disadvantages or risks specifically associated with taking part in the study. The associated risks of your routine surgery will have been discussed separately with you by your consultant.

**What are the possible benefits of taking part?**

There is no clinical benefit from taking part in the study and it will not affect the outcome of your surgery. The study endeavors to develop a drug therapy for the treatment for Dupuytren's disease.

**Will my taking part in the study be kept confidential?**

All information which may be collected will be kept strictly confidential. Any information about you which leaves the hospital will have your name, age, address and all identifiable information (including patient/hospital/NHS number) removed so that you cannot be recognised from it.

**What will happen to the results of the study?**

The results of the study will be submitted for publication in scientific journals. You may receive information about the results of the study upon request.

**Who has reviewed the study?**

The study has been reviewed by the Lancashire Teaching Hospitals Trust Ethics Committee.

**Contact for further information:**

Dr Christopher Stephen  
Tel: 01772 716565 (Royal Preston Hospital)  
Email: Christopher.Stephen@lthtr.nhs.uk

# Dupuytren's Disease Study Consent Form

Researchers: Mr C Stephen Dr L Touil Mr SW McKirdy Prof J Singh

Please read carefully before initialing each statement and finally signing and dating the bottom

I .....can confirm that I have received, read and understood the information sheet for the Effects of ACE Inhibitors in Dupuytren's Disease Study (V1/Aug 09). I have been given the opportunity to discuss the study with anyone I wish, including the researchers, prior to giving my consent. I have been given the opportunity to have my questions answered.

**Initials.....**

I understand that my participation is entirely voluntary and I am free to withdraw my consent at any point without explanation without affecting my medical care or legal rights.

**Initials.....**

I give permission for access to my medical notes by responsible individuals from Lancashire Teaching Hospitals NHS Foundation Trust or regulatory authorities in connection with this study.

**Initials.....**

I agree to participate in the Use of ACE Inhibitors in Dupuytren's Disease Study.

**Initials.....**

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**Name of Patient**  
**Block Capitals**

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**Signature**

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**Date**

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**Name of Person taking consent**  
**Block Capitals**

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**Signature**

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**Date**

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**Name of Researcher**  
**Block Capitals**

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**Signature**

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**Date**

1 copy to patient and 1 copy to researcher  
Original to be placed in notes

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